Acta Haematologica

International Journal of Haematology   Journal International d'Hématologie   Internationale Zeitschrift für Hämatologie

Editors-in-Chief H. Lüdin, Basel, und G. Rosenow New York

Organe Officiel de la Société Européenne d'Hématologie

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Vol. 32

Printed in Switzerland by Buchdruckerei Aargauer Tagblatt AG, Aarau
Clichés: Steiner & Co. AG, Basel

Acta haemat. 32, 1–8 (1964).

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Ferrokinetics in Pyridoxine-Responsive Anaemia

By Stuart Roath* Martin S. Bourne and M. C. G Israëls

The use of radio-iron in the study of iron metabolism has shown patterns which are characteristic for some kinds of anaemias whose mechanisms may be better understood by the use of this technique. The pyridoxine responsive anaemias, of which there are only 23 cases reported, including ours, still lack a clear explantation of their defective iron handling and aetiology For this reason ferrokinetic studies were performed in the five subjects described and the significance of these studies discussed with regard to this particular entity

Material

Patient P P was reported by Dawson et al. (8) J W his cousin, is confirmed case of pyridoxine-responsive anaemia with good response to pyridoxine. C. S. P and H. G. P are brothers of P P and H. H. is another cousin of P P Their pertinent haematological findings at the time of this report are summarized in table I. This family is fully reported by Bourne et al. (6)

Methods

2 ml of solution of Fe^{59} corresponding to an activity of approximately 4 µc. was administered to each of the four subjects according to the method described by Loeffler et al. (16) Samples were withdrawn immediately before the administration of the isotope and at suitable intervals afterwards to calculate the rate of plasma iron clearance (T½ value is given; normal 60-120 minutes) as carried out by Huff et al. (13).

Samples of peripheral blood were taken at intervals of few days over the subsequent three weeks and from these and the red-cell volume, the percentage utilisation of the injected iron was determined utilisation curves for each subject were also plotted as described by Dubach et al. (9) and Finch et al. (10)

The plasma iron turnover percentage daily replacement of haemoglobin and the red blood-cell life span were also determined; the calculation being based on the work of Huff et al. (13) and Finch et al. (10). Serum iron estimations were carried out on pooled samples by the method of Ramsay (17).

Sybil Mary Pilkington Research Fellow

Results

Plasma Iron Clearance All 5 subjects times fell into the range 60–104 minutes, so they must therefore be regarded as having an approximately normal value for this investigation. These results, shown in fig. 1 fail to demonstrate any relationship between the severity of the anaemia, or the serum iron level, and the T½ obtained.

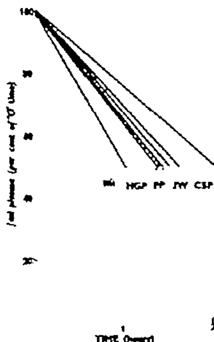


Fig. 1 Plasma iron clearance curves.

Utilization of Iron. Fig. 2 shows the utilization curves for all 5 subjects, plotted as a percentage of the injected dose of radio-activity after correction for radioactive decay. The shape of the curve follows the normal pattern in each case, but the two subjects who had had frank anaemia failed to utilize the normal percentage of iron, though the ten-day values in both cases were more than 65 %, and one, H. H., showed an even lower percentage utilization despite a relatively high haemoglobin.

The other ferrokinetic data is shown in table II a progression from approximately normal levels in C.S.P. to markedly abnormal

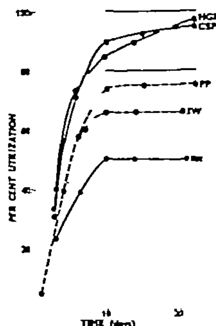


Fig. .. Percentage iron utilization curves.

findings in J W is seen H.G.P., who had never been found to have overt symptoms of anaemia, also had an unusually high Fe clearance, haemoglobin daily replacement and low red blood-cell life-span by the methods used.

Discussion

The plasma-iron-clearance is regarded as indication of the rate of transfer of iron to red cells and, in the presence of an adequate iron pool, some guide to the rate of erythropoiesis, be thus effective red cell production or not. Therefore, the normal levels obtained in our investigations suggest that the rate of erythropoiesis, or at least iron incorporation into the erythrocytes, is not grossly altered. Though this in itself does not give us any positive information regarding the mechanism of this type of anaemia it suggests that the defect is dissimilar to that in those conditions where the $T\frac{1}{2}$ is shortened, as it is in haemolytic anaemias (including thalassaemia) and pernicious anaemia. The refractory anaemias, have a wide range of values, so that although our cases had plasma iron clear ance times compatible with some of those obtained in these types

of anaemia, this is such a heterogenous group that classification within this category is not helpful.

Plasma iron clearance in other reported cases of pyridoxine responsive anaemias are demonstrated in table III and will be seen to be generally shorter than normal, with some variation within the small number of investigations done.

Table I
Haematological findings in studied members of family

	Haemoglobin range grams per 100 ml	Packed cell volume (lowest) %	Mean corpuscular haemoglobin concentration %	Serum iron before and during treatment µg %	Maximum serum iron saturation percentage
C. S. P	12.0	42	28.5	320–180	90
H. C. P	12.8	39	32.0	250–110	60
P P	5.2–13.6	19	27.5	220–130	60
J W	9.5–13.7	36	30.3	290– 80	85
H. H.	11.8–14 0	37	32.0	290–210	95

Table II
Ferrokinetic data obtained from iron turnover and serum iron estimations.

	Daily iron clearance/100 ml		Haemoglobin formation/100 ml	Daily replacement Hb. %	R. B. C. life span days
	Plasma mg	Whole blood mg	Whole blood g		
C. S. F	0.67	0.39	0.12	1.0	100
H. G. P	0.77	0.41	0.125	0.93	103
P P	0.89	0.53	0.16	1.23	80
J W	1 10	0.68	0.20	1 71	58
H. H.	0.96	0.39	0.084	0.72	139

Similarly iron utilization in our 5 subjects was in the 49–100% range, which shows a range of deviation from normal values, but does not resemble the 15–30% range obtained by DACIE et al. in their cases of refractory anaemia or the very low percentages reported by some authors quoted in table III except HAVARD (11) whose patient had 80 % utilization.

The red-cell survival times obtained, calculated on a reciprocal basis from the percentage daily replacement of haemoglobin were found to show slight shortening in the two siblings and a greater degree of shortening in P P and J W the clinically affected subjects. P P however had previously had a survival time measured by the Cr^{51} method which gave an apparently normal result (8) The red-cell survival times shown in table III are shortened in 4 cases—one markedly so—and normal in one subject.

Table III

Ferrokinetic data reported by other authors.

Author	T½ Plasma iron clearance minutes	Iron turnover	% Red-cell iron utilization	Red-cell survival
Jones and Hutt (15)	42	5 mg/100 ml plasma/day	35 (day 7) 47 (max.)	T = 19½ days (Cr 51)
Vuylsteke et al. (20)	46	4.3 mg/100 ml plasma/day	10 (max.)	—
Verloop and Rademaker (19)	40	5.2 mg/100 ml whole blood/day	17 (max.)	T½ = 18 days
Bernard et al. (1)	42	3.4 mg/100 ml plasma/day	12	—
Bishop and Bethell (3)	47	235 mg/day (? Plasma)	2 3	—
Bickers et al. (2)	37	7.3 mg/100 ml blood/day	—	T½ (Cr 51) 14 – 17 days
Havard (11)	normal"	normal"	80 (day 9)	T½ (Cr 51) = 21 days
Bottomley (case 1) (5)	36	—	15 (day 10)	T½ = 10 days
Normal values	60–120	0.74–0.8 mg/100 ml/plasma/day	80	Cr 51 T½ = 28 days

The values obtained for this investigation, using the method described, depend ultimately on the current plasma iron level as an essential part of the calculation. Though at the time of study this level was consistently over 200 mg per 100 ml, at other times random plasma iron estimations have shown markedly differing, though still abnormal, values. In view of these observations we would hesitate to rely on results obtained from so variable an estimation, as has been noted by Bothwell and Mallett (4) and Sterngle and Schade (18) who comment on the effect of even diurnal variations of plasma-iron level and the consequent apparent quantitative changes in iron turnover estimations obtained.

We would therefore have to regard our red-cell survival times, daily iron clearance and haemoglobin replacement values as being subject to an unknown variation which pooling of plasma samples for iron determinations might not effectively correct. On the other hand, the plasma-iron clearance and red-cell iron utilization are not dependent on a value (plasma iron) which is known to be variable, and many therefore be of more significance.

From the results obtained in our subjects we are unable to show a uniform aberration of iron handling despite the abnormally high plasma iron values obtained in all cases, history of severe anaemia, in two instances and suggestive haematologic findings in their relatives investigated. The other cases of pyridoxine responsive

anaemia in which similar studies have been done tend to show a varying degree of shortening of the T½ for plasma iron clearance and an impairment of red-cell iron utilization which may be marked (3) or moderate (15) HAVARD (11) described a case with similar ferrokinetics to some of ours, which had a similar complete response to pyridoxine therapy while it is of interest to note that those cases showing abnormal patterns of iron handling had an incomplete response, their haemoglobin levels usually remaining below 10 G%

These findings might be interpreted to show that pyrodixine responsive anemia may be a heterogenous entity and that either more than one defect occurs—those responding completely and incompletely may be examples of dissimilar types—or that the defect is due to a genetically controlled aberration of different penetrance, which the variable abnormalities in the 3 brothers in our series would support.

An alternative theory would be the appearance of a permanent irreversible, or only partly reversible, defect in iron-handling following the long standing establishment of the abnormality present in pyridoxine-sensitive states. This irreversible state may simply be due due to prolonged iron loading itself, producing a condition of further interference with iron utilization in erythropoiesis. Whatever the cause of the "irreversible state" it would seem that the subjects reported as having a complete response to pyridoxine have not arrived at this point, and therefore, like our cases, could be expected to have fairly normal ferrokinetics.

Conclusions

It appears that pyridoxine responsive anaemia is a state capable of showing considerable differences in iron-handling between different subjects studied by ourselves and other authors. Both aetiological hypotheses outlined can be supported by the investigational findings, but the infrequent occurrence of this syndrome makes large scale studies to delineate the aberration of iron metabolism difficult to perform. It does seem possible that more than one type of defect may be found or that there might be a differing degree of abnormality in many of the subjects found with this type of anaemia.

Summary

Radio iron studies have been carried out on 5 members of one family who have pyridoxine-responsive anaemia. The plasma iron clearances were within the normal range. The utilization of iron was abnormal in some. These results are discussed and compared with those in similar cases described in the literature. It is suggested that pyridoxine-responsive anaemia may be heterogenous group or that the defect may become irreversible in time.

Résumé

Des études à l'aide de fer radioactif ont été faites chez 5 malades d'une famille atteints d'une anémie sensible à la pyrodixine. La clearance du fer plasmatique est normale. Quelques membres montrent une utilisation pathologique du fer. Les résultats sont discutés et comparés aux résultats obtenus chez des cas semblables rapportés dans la littérature. Les auteurs suggèrent que l'anémie sensible à la pyridoxine est un groupe hétérogène d'anémies et que le défaut peut devenir irréversible avec le temps.

Zusammenfassung

Bei 5 Mitgliedern einer Familie mit Pyridoxin-empfindlicher Anämie wurden U ntersuchungen mit radioaktivem Eisen vorgenommen. Die Plasmaeisen-Clearance war normal. Bei Einigen war die Eisenverwertung pathologisch. Die Resultate werden besprochen und mit den bei ähnlichen Fällen in der Literatur mitgeteilten Befunden verglichen. Es wird vermutet, daß die Pyridoxin-empfindliche Anämie eine heterogene Gruppe darstellt oder daß der Defekt mit der Zeit irreversibel werden kann.

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Acta haemat. 32: 9–16 (1964)

Aus der Medizinischen Universitätspoliklinik Basel (Direktor: Prof. Dr. O. GSELL), der Universitätskinderklinik Groningen (Direktor: Prof. Dr. J. H. P. JONXIS) und der Medizinischen Abteilung des Bezirksspitals Interlaken (Chefarzt: PD Dr. P. COTTIER)

Eine neue Hämoglobin I-Variante: Hb $I_{Interlaken}$

Von H. R. MARTI, C. PIK und P. MOSIMANN

Hämoglobin I (Hb I) ist ein sehr seltenes anomales Hämoglobin, das 1955 von JENSEN et al. (11) und CABANNES et al. (6) erstmals beschrieben wurde. Die Bezeichnung Hb I stammt von RUCKNAGEL et al. (18), die im gleichen Jahr die gefundene Variante als neues Hämoglobin identifizierten. Seither wurden weitere Hb I-Fälle von SCHWARTZ (19), BOULARD (5), RANNEY (17) und THOMPSON et al. (20) beobachtet. MURAYAMA (14) konnte die Anomalie 1962 bei einem der von SCHWARTZ et al. (19) gefundenen Träger lokalisieren. In der α-Polypeptidkette ist in Stellung 16 ein Lysin durch Asparaginsäure ersetzt. THOMPSON et al. (20) fanden 1963 bei ihrem Fall die gleiche Substitution. Die folgende Mitteilung berichtet über einen in der Schweiz beobachteten Patienten italienischer Herkunft mit einer neuen Hb I-Variante.

Kasuistik

C. C. ♂ geb. 1942. Vom 20. 12. 1962 – 6. 3. 1963 in der Medizinischen Abteilung des Bezirksspitals Interlaken (Schweiz) hospitalisiert.

Familienanamnese: Die Familie des Patienten stammt aus Frasso (Benevent, Süditalien) und aus Rom. Die Eltern und 5 Geschwister des Probanden sind gesund. In der Familie sind keine Fälle von Ikterus oder Anämie bekannt.

Persönliche Anamnese: Der Patient war bis zu Beginn des jetzigen Leidens nie ernstlich krank, klagte jedoch seit Jahren über leichte Fettunverträglichkeit. Er erhielt nie Bluttransfusionen und seit 8 Jahren nie irgendwelche Injektionen und hatte keinen Kontakt mit Fällen von Hepatitis.

Am 17. 12. 1962 fühlte er sich abnorm müde und bemerkte am 18. 12. gelbe Skleren und eine Dunkelfärbung des Urins. Am 20. 12. wurde der Mann mit der Diagnose Hepatitis epidemica hospitalisiert.

Klinische Befunde: Guter Allgemein- und Ernährungszustand, Temperatur afebril. Mittelstarker generalisierter Ikterus. Herz und Lungen ohne pathologischen Befund.

Blutdruck 120/70 mm. Leber leicht vergrößert, wenig druckdolent. Milz nicht palpabel. Leicht vergrößerte Lymphknoten axillär beidseits.

Allgemeine Laboratoriumsuntersuchungen. Senkungsreaktion 2/4 mm in der 1. resp. 2. Stunde. Serumbilirubin gesamt 12,5 mg% direkt 8 mg%. Serumeisen 314 γ%. Transaminasen SGPT 1760 E, SGOT 114 E, Prothrombin nach Quick 50 %, alkalische Phosphatase 10,3 E. Leukocyten 6200 mit normaler Verteilung. Im Urin Bilirubin stark positiv.

Diagnose, Therapie und Verlauf. Die oben angegebenen Befunde bestätigten die Einweisungsdiagnose Hepatitis epidemica. Schon beim Spitaleintritt ließen vereinzelt Schießscheibenzellen und basophil punktierte Erythrocyten im Blutbild zusammen mit der Herkunft des Patienten aus Süditalien an eine Hämoglobinopathie denken. Die Therapie der Hepatitis bestand in Bettruhe, fettarmer Diät, Infusionen von Lävulose mit den Vitaminen B und C sowie Bilamid. Der Icterus nahm zunächst noch etwas zu, das Bilirubin erreichte am 3. 1. 1963 23,4 mg%, davon 18,4 mg% direktes Bilirubin, die Prothrombinwerte nach Quick schwankten zwischen 12–62 %, die Milz wurde vorübergehend palpabel. Der Patient erhielt in der Folge vom 14.–28. 1. 1963 30 mg Prednison täglich per os, dann wurde die Dosis bis zum 6. 2. schrittweise abgebaut. Während dieser Zeit sanken die Bilirubinwerte rasch ab. Am 8. 2. betrug das gesamte Bilirubin 2,1 mg%, das direkte 1,1 mg%. Die Temperatur war afebril, und die Senkungsreaktion blieb auf normalen Werten.

Einige Wochen lang war noch eine leichte Hyperbilirubinämie vorhanden. Beim Spitalaustritt wurden folgende Befunde erhoben: Bilirubin gesamt 0,83 mg%, direkt 0,34 mg%. Bromsulfaleinretention mit 8,7 % normal, Prothrombin 100 %. Serumeiweiß 6,8 g%, davon 25 % γ-Globuline. Leber und Milz nicht mehr vergrößert, keine vergrößerten Lymphknoten.

Hämatologische Untersuchungen

Technik. Zur Bestimmung von Hb F wurde die von Betke et al. (3) angegebene Modifikation der Singerschen Alkalidenaturierung verwendet. Der Nachweis der übrigen Hämoglobine erfolgte mittels Stärkeblockelektrophorese nach dem von Kunkel und Wallenius (12) angegebenen Prinzip. Methämoglobin wurde nach der Methode von Evelyn und Malloy (7) bestimmt und der Heinzkörper Test wurde nach der Methode von Beutler et al. (4) ausgeführt. Alle verwendeten Untersuchungsmethoden wurden kürzlich an anderer Stelle ausführlich beschrieben (13).

Die Untersuchung des Blutes ergab folgende Werte: Hämoglobinkonzentration 13,5 g%, Erythrocyten 4,4 Millionen/mm³, Hb_E 31 $\mu\mu g$, Reticulocyten 1?–40 ‰, Hb F 0,41 %, Hb A_2 1,5 %. Im Hämolysat des Probanden fand sich in der Stärkeblockelektrophorese bei pH 8,6 eine schnell wandernde anomale Fraktion, die 23 % des gesamten Hämoglobins ausmachte (Abb. 1). Die Wanderungsgeschwindigkeit lag zwischen derjenigen von Hb J und Hb H. Bei pH 6,5 wanderte das anomale Hämoglobin zur Kathode und trennte sich erst nach mehrstündiger Laufzeit von Hb A_1 (Abb. 2).

In den Erythrocyten waren mit Nilblausulfat auch nach Inkubation bei 37° C keine Innenkörper nachweisbar. Das anomale Hb unterschied sich in der Hitzeresistenz, Säureresistenz und in der

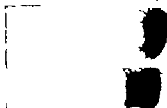
Start HbA₂ HbA₁ Hb $I_{Interl.}$



Wanderungsrichtung zur Anode

Abb. 1. Stärkeblockelektrophorese pH 8,6. Veronal/Veronal-Na-Puffer 0,075 M 600 V 60 mA 12 Std. + 4 °C. Oben = Hämolysat eines normalen Erwachsenen unten = Hämolysat des Probanden.

Start Hb $I_{Int.}$ Hb A_1



Wanderungsrichtung zur Kathode

Abb. 2. Stärkeblockelektrophorese pH 6,5. Phosphat Puffer 0,07 M 600 V 94 mA 12 Std. + 4 °C. Oben = Hämolysat eines normalen Erwachsenen unten = Hämolysat des Probanden.

Geschwindigkeit der Alkalidenaturierung nicht von Hb A_1 Der Sichelzelltest fiel negativ aus. Frisches Citratblut des Probanden enthielt kein Methämoglobin (Met Hb) eine 6 Tage bei +4 C aufbewahrte Blutprobe wies 0,26 / Met Hb auf. Im Hämolysat waren nach 6 Tagen 0,55 / und nach 13 Tagen 1 15 % Met Hb vorhanden. Das anomale Hb zeigte bei +4 C keine gesteigerte Spontanoxydation in Met Hb Hingegen kam es bei Inkubation der Erythrocyten mit Acetylphenylhydrazin bei 37 C bereits nach 30 Min. zur Innenkörperbildung und nach einer Stunde waren erheblich mehr Innenkörper vorhanden als in normalen Kontrollerythrocyten (Abb 3) Die Glucose-6-phosphatdehydrogenase Aktivität erwies sich bei spektrophotometrischer TPNH Messung (13) mit 10 7 E als normal.

Identifizierung des anomalen Hämoglobins (Universitätskinderklinik Groningen) Das Hämolysat wurde gegen Phosphatpuffer 0 05 M



Abb 3. *Innenkörperbildung* bei Inkubation der Erythrocyten mit Acetylphenylhydrazin während 1 Std. Links = Erythrocyten eines normalen Erwachsenen rechts = Erythrocyten des Probanden.

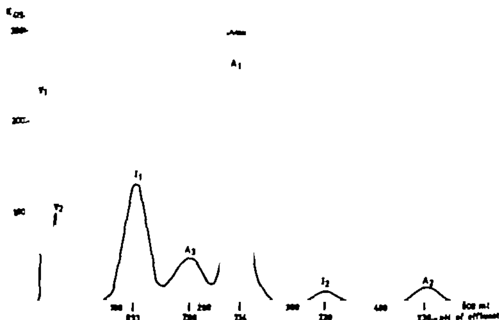


Abb 4 Trennung der Hämoglobine durch *Carboxymethylcellulose*-
Zwischen Hb A und Hb A_2 ist die anomale Minorfraktion Hb I_2 zu erkennen

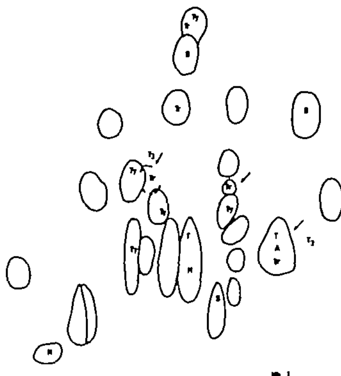


Abb. 5. Fingerprint der isolierten anomalen Hauptfraktion. A = Arginin, H = Histidin, T = Tryptophan, Ty = Tyrosin, S = Schwefel.

pH 6,5 dialysiert und mittels Carboxymethylcellulose-Säulenchromatographie mit einem Phosphatpuffer-Gradienten nach der Methode von HUISMAN et al. (8) fraktioniert. Zu Beginn wurde das anomale Hämoglobin eluiert; zwischen Hb A_1 und Hb A_2 erschien eine kleine Fraktion Hb I_2, das die Zusammensetzung $\alpha^I_2\delta_2$ besitzt (1) und weniger als 0,5 % des gesamten Hb ausmacht (Abb. 4).

Die konzentrierte anomale Hauptfraktion wurde nach Trypsinspaltung mit der Fingerabdruckmethode von INGRAM (9, 10) analysiert (Abb. 5). Die normalerweise intensive Tryptophanreaktion im Peptid αT_3 fehlte weitgehend; dafür waren 2 neue Tryptophanflecken zu erkennen, einer an der Stelle des Peptids αT_4, entsprechend dem Befund von MURAYAMA UND INGRAM (15), und einer in der neutralen Zone. Die Analyse der Aminosäurensequenz des anomalen Peptids an der Stelle αT_4 ergab zwar ebenfalls eine Substitution durch Asparagin, aber nicht in Stellung 16, wie bei dem von MURAYAMA (14) und THOMPSON et al. (20) untersuchten Hb I,

Tabelle I

		T				αT			
ala –	ala –	try –	gly –	lys –	val	gly –	ala –	his –	ala –
– ala	ala –	try –	gly –	asp –	val –	gly –	ala –	his –	ala
– ala –	ala –	try –	asp –	lys –	val –	gly –	ala –	his –	ala –
12	13	14	15	16	17	18	19	20	21

Zusammensetzung der Peptide αT *und* αT bei Hb A_1 Hb I und Hb $I_{Interlaken}$
oben = Hb A_1 Mitte = Hb I unten = Hb $I_{Interlaken}$

sondern in Stellung 15 (Tab 1) Auch diese Substitution erschwert die Spaltung zwischen den Peptiden αT_3 und αT_4 Das Peptid in der neutralen Zone muß das abnorme αT_3 enthalten die vorhandene Menge reichte für eine weitere Analyse nicht aus.

Diskussion

Wie die von Rucknagel et al. (18) Portier et al. (16) und Thompson et al. (20) beobachteten heterozygoten Träger der Hb I Anomalie, so zeigte auch der hier beschriebene Patient keine klinischen Erscheinungen der Hämoglobinanomalie Hämoglobinkonzentration und Blutbild waren praktisch normal, einzig die Reticulocytenzahl war leicht erhöht.

In den Erythrozyten ließ sich in vitro eine vermehrte Innenkörperbildung durch Phenylhydrazin nachweisen, im übrigen lagen aber keine weitern Anhaltspunkte für eine besondere Instabilität des anomalen Hämoglobins vor Die im Blut zirkulierenden Erythrocyten enthielten keine Innenkörper und es war in vivo und in vitro keine gesteigerte Met Hb-Bildung erkennbar Im Gegensatz zu der von Atwater et al. (2) beschriebenen Hb I Thalassämie mit 70 % Hb I bildeten die Erythrocyten dieses Patienten bei Inkubation mit 2 % $Na_2S_2O_4$ keine Sichelzellen.

Das anomale Hämoglobin wandert bei pH 8,6 etwas weniger rasch als Hb I die Untersuchung mit der Fingerabdruckmethode ergibt aber dieselben anomalen Peptide, und die Substitution erfolgt in der α-Kette beide Male durch Asparaginsäure, beim klassischen Hb I in Stellung 16 und hier in Stellung 15 Trotz der Differenz der elektrophoretischen Wanderungsgeschwindigkeit erscheint es sinnvoll, das neue anomale Hämoglobin als Hb I Variante aufzufassen Wir bezeichnen es gemäß heutiger Gepflogenheit nach dem Ort der Hospitalisation des Trägers als «Hb $I_{Interlaken}$»

Zusammenfassung

ähriger aus Süditalien stammender Patient wurde wegen Hepatitis epide
al Interlaken (Schweiz) hospitalisiert. Es bestand keine Anämie, hingegen
Blutausstrich erstmals Schießscheibenzellen und die Zahl der Reticulocy-
t erhöht. Die Hämoglobinelektrophorese ergab als überraschenden Befund
ei pH 8,6 schnell wandernden anomalen Hämoglobins, das als Hb I Va-
fiziert werden konnte. Im Gegensatz zum Hb I, dessen Formel $\alpha_2^{16\,Asp}\beta_2$
let sich die Substitution in der neuen Variante in Stellung 15 Das neue
wird als Hb I$_{Interlaken}$ bezeichnet.

Summary

ears old white male of Italian origin hospitalized for infectious hepatitis
town of Interlaken is described. No anaemia was present, however target
htly increased reticulocytes were the indication for electrophoretic separation
bin. 23 % of fast moving abnormal haemoglobin was found. At pH 8 6
l fraction migrated between Hb J and Hb H and was identified as new
Hb I. In contrast to the classical Hb I with the formula $\alpha_2^{16\,Asp}\beta_2$ the new
umed the same substitution in position 15 of the α-chain $\alpha_2^{15\,Asp}\beta_2$. It was
signation of "Hb I$_{Interlaken}$

Résumé

lien du sud, agé de 20 ans, est hospitalisé à Interlaken (Suisse) pour une
ctereuse. Le malade est pas anémique mais son frottis sanguin révèle
lules en cibles et une légère augmentation des réticulocytes. Contre toute
ctrophorèse de l'hémoglobine met en évidence 23% d'une hémoglobine
idement à un pH de 8,6 et qui est identifiée comme une variante de l'Hb I
ast à l'Hb I dont la formule est $\alpha_2^{16\,Asp}\beta_2$, la nouvelle variante est caracté-
e substitution en position 15. Cette nouvelle hémoglobine, Hb $\alpha_2^{15\,Asp}\beta_2$
de Hb I$_{Interlaken}$ ».

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Acta haemat. 32: 17–26 (1964)

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Histochemical Studies of Leukocytes from an Inflammatory Exudate

VI. Demonstration of Non-Diaphorase-Coupled Dehydrogenase Activity Using Phenazine Methosulphate*

By Henrik R. Wulff

In a previous study (17) the activity patterns of DPN and TPN linked dehydrogenases** in inflammatory leukocytes were recorded using standard histochemical reactions.

The leukocytes were incubated in a medium containing the substrate of the dehydrogenase, the coenzyme (DPN or TPN) and a tetrazolium salt. Electrons were transferred from substrate via dehydrogenase to coenzyme and then to tetrazolium by way of DPN- or TPN-diaphorase. Thus, the standard reactions, which in the present paper are referred to as *dehydrogenase-diaphorase reactions*, depended both on activity of the dehydrogenase in question and on the diaphorase. The nature of DPN and TPN-diaphorase is controversial. Previously they were believed to be identical with DPNH- and TPNH-cytochrome reductase (8) but recent findings suggest that at least DPNH cytochrome reductase is an artifact and that several carriers are involved in the electron transport from DPNH to the cytochrome system, including DPNH dehydrogenase (2) and possibly ubiquinones. It is uncertain at what point in the respiratory chain tetrazolium intercepts the electron transfer. DPNH dehydrogenase does not reduce redox dyes, but ubiquinones have been shown to reduce tetrazolium (14). Lipoyl dehydrogenase (Straub diaphorase) (9), which is not a component of the respiratory chain, can also transfer electrons from DPNH to tetrazolium, and may therefore contribute to the histochemical DPN-diaphorase reaction. In inflammatory leukocytes, however the DPN- and TPN-diaphorase reactions present identical activity patterns (16) and probably both reactions reveal a part of the respiratory chain.

Coenzyme linked dehydrogenases may also be demonstrated in the absence of diaphorase, if phenazine methosulphate (PMS) is used as electron carrier between coenzyme and tetrazolium instead of indigenous diaphorase. Such *dehydrogenase-PMS reactions* are used routinely for the detection of dehydrogenases after electrophoresis

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* Aided by a grant from Arvid Nilsson's Fond.

** The following abbreviations are used: DPN and TPN, di- and triphosphopyridine nucleotide; DPNH and TPNH, reduced form of DPN and TPN; PMS, phenazine methosulphate (N-methyl-phenazonium methosulphate).

(7) but have only recently been employed histochemically by VAN WIJHE et al (15) in studies of guinea pig skeletal muscle. They found that the use of PMS increased the staining for DPN linked lactic and α-glycerophosphate dehydrogenase in the white muscle fibres, and concluded that a low level of DPN-diaphorase in these fibres, which possess a predominantly glycolytic metabolism, had been a limiting factor in the dehydrogenase-diaphorase reactions. Leukocyte metabolism is also chiefly glycolytic, and it was therefore decided to re-examine the activity of a number of dehydrogenases in inflammatory leukocytes using dehydrogenase PMS reactions.

Methods

The studies were an exact repetition of the previously reported dehydrogenase experiments (17) apart from the addition of PMS to the incubation media. Successive samples of migrating leukocytes were obtained using the 'skin window' technique (11). The leucocytes migrated from small excoriation to the undersurface of coverslip, which was replaced hourly for 12 hours. The coverslip preparations were dried in air for few minutes before incubation for 30 minutes at 37 °C in PEARSE dehydrogenase media (10), to which had been added 0.1 ml 1 PMS solution per 1 ml medium. The tetrazolium salt employed was Nitro BT and the final coenzyme concentration was 0.01 M (or in few experiments, 0.001 M) The PMS-containing media were protected from light and were stable for 2 hours, when stored at 4 C. Control preparations were incubated in media without PMS and in PMS-containing media from which the substrate had been omitted. The following experiments were made TPN-linked glucose-6-phosphate dehydrogenase (4 skin window' experiments) TPN-linked isocitric dehydrogenase (4 skin window' experiments) TPN-linked malic dehydrogenase (2 'skin window' experiments), DPN-linked lactic dehydrogenase (3 'skin window' experiments) DPN-linked malic dehydrogenase (3 'skin-window experiments) and DPN-linked isocitric dehydrogenase (3 'skin-window experiments) The experiments were made on two healthy subjects.

Results

Dehydrogenase activity was revealed by the precipitation of formazan in the cytoplasm of the emigrated cells, and the degree of activity was roughly graded on a "none to 'very strong" basis. The results are recorded in table I beside the results previously obtained using the dehydrogenase-diaphorase reactions. The formazan deposits usually formed coarse granules in the neutrophils and finer granules in the macrophages, but in intensely stained cells of either type the precipitation was almost diffuse

The staining intensity obtained using the dehydrogenase-PMS reactions varied considerably in different preparations stained for the same enzyme and even in different areas of the same prepa



Fig 1) Glucose-6-phosphate dehydrogenase PMS reaction. Very strong staining of neutrophils from early skin window' preparation. b) Same reaction. Strong staining *of neutrophils and macrophages from late skin window' preparation.*) *Glucose-6-* phosphate dehydrogenase-diaphorase reaction. Two neutrophils showing moderate and ery weak staining reaction.

ration, but in spite of this variability it is evident that the enzymes showed completely new activity patterns in the presence of PMS The dehydrogenase-diaphorase reactions had always given constant results.

No consistent difference was observed between the activity of inflammatory cells from early and late skin window preparations and all enzymes showed equal activity in neutrophils and macrophages. The few eosinophils encountered in the preparations, however were less strongly stained than the surrounding cells (fig 2b) In contrast, the dehydrogenase-diaphorase reactions had revealed a gradual increase in enzyme activity from early to late preparations, and the macrophages had been much more strongly stained than the neutrophils. Only the glucose-6-phosphate dehydrogenase-diaphorase reaction had presented a different pattern a minority of the granulocytes had shown a moderate activity *whereas the remainder was practically unstained.*

The most outstanding finding however was the difference in the staining intensity given by the different enzymes. The dehydrogenase PMS reactions revealed much greater activity of *TPN linked glucose-6-phosphate and isocitric dehydrogenases* in both neutrophils and macrophages than the conventional reactions (fig 1 and 2a, d) In the case of *DPN-linked isocitric dehydrogenase* on the other hand

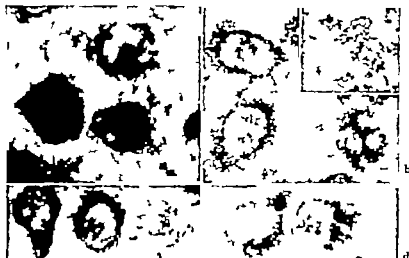


Fig. 2. a) TPN-linked isocitric dehydrogenase PMS reaction. Strong staining of two macrophages and a neutrophil. b) DPN-linked isocitric dehydrogenase PMS reaction. Moderate staining of two macrophages and a neutrophil. Inset: Weak staining of an eosinophil (a few formazan granules are seen between the specific granules). c) DPN-linked isocitric dehydrogenase-diaphorase reaction. Strong staining of two macrophages and moderate staining of a neutrophil. d) TPN-linked isocitric dehydrogenase-diaphorase reaction. Moderate staining of two macrophages from late skin window preparation.

addition of PMS to the medium had the opposite effect (fig. 2b, c). The dehydrogenase-diaphorase reaction for this enzyme had shown great activity in the macrophages and moderate activity in the neutrophils, but the dehydrogenase PMS reaction never gave more than a moderate staining of both cell types. This pattern was also seen in the case of *DPN-linked malic dehydrogenase*, whereas little *TPN-linked malic dehydrogenase* activity was observed using either reaction. The *lactic dehydrogenase* PMS reaction showed greater activity than the dehydrogenase PMS reactions for the two other DPN linked enzymes (fig. 3); in the most strongly stained preparations the staining of the neutrophils was stronger and that of the macrophages only slightly weaker than the staining obtained using the conventional media. The variability of the dehydrogenase-PMS reaction for this enzyme was pronounced, and in some preparations the cells only contained few formazan granules.

During incubation a blue formazan precipitate was formed in the PMS-containing media covering the coverslip preparations, particularly in experiments for glucose-6-phosphate, TPN linked

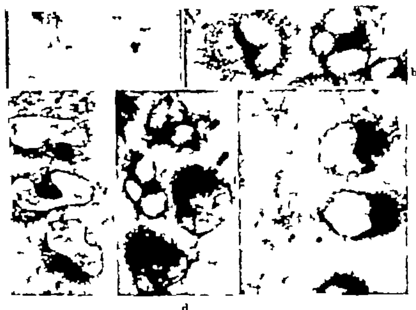


Fig 3. a) Lactic dehydrogenase-PMS reaction. Weak staining of two neutrophils. b) Same reaction. Moderate staining of two neutrophils. c) Same reaction. Moderate staining of three macrophages. d) Same reaction. Strong staining of neutrophils and macrophages. e) Lactic dehydrogenase-diaphorase reaction. Very strong staining of macrophages and moderate staining of neutrophils from late "skin window" preparation.

isocitric, and DPN linked lactic dehydrogenase, whereas the incubation media without PMS always remained clear

The inflammatory cells from control preparations incubated in the different PMS-containing media without specific subtrates remained unstained or only contained very few formazan granules, whereas those incubated in the media without PMS showed the expected dehydrogenase-diaphorase activity

Comment

The observation that the same dehydrogenases present completely different activity patterns in inflammatory cells, when the dehydrogenase PMS reactions are used instead of the dehydrogenase-diaphorase reactions, suggests that the two types of reactions demonstrate different fractions of these dehydrogenases.

The dehydrogenase PMS reaction reveals great activity of glucose-6-phosphate dehydrogenase in all inflammatory cells, and it is therefore necessary to explain, why the corresponding dehy

Table I

Enzyme	Dehydrogenase PMS reaction: neutrophils and macrophages	Dehydrogenase-diaphorase reaction: neutrophils	Dehydrogenase-diaphorase reaction: macrophages
TPN-linked glucose-6-phosphate dehydrogenase (fig. 1)	++ — ++++	(+) or ++	+ → ++
TPN-linked isocitric dehydrogenase (fig. 2 and d)	++ — +++	0 → +	+ → ++
DPN-linked lactic dehydrogenase (fig. 3)	+ — +++	+ → ++	+++ → ++++
DPN-linked malic dehydrogenase	+ — ++	+ → ++	+++ → ++++
DPN-linked isocitric dehydrogenase (fig. 2b and)	+ — ++	+ → ++	+++ → ++++
TPN-linked malic dehydrogenase	0 — +	0 → (+)	(+) → +

The degree of activity revealed by the dehydrogenase-PMS and the dehydrogenase diaphorase reactions has been graded arbitrarily from 'none' to 'very strong' (0 none, (+) very weak, + weak, ++ moderate, +++ strong, ++++ very strong). The results obtained using the dehydrogenase PMS reactions varied within the recorded limits. The dehydrogenase-diaphorase reactions showed no such variations, but the staining of the inflammatory cells increased regularly from early to late skin window preparations. Only the glucose-6-phosphate dehydrogenase-diaphorase reaction presented different pattern. For further details see text.

drogenase-diaphorase reaction showed almost no activity in the majority of the granulocytes and only a moderate activity in the remainder of the cells. Previously it was demonstrated that all inflammatory cells present a degree of TPN-diaphorase activity which equals that of DPN-diaphorase (16) and the weakness of the dehydrogenase-diaphorase reaction is therefore neither due to a lack of the dehydrogenase nor to a lack of TPN-diaphorase, but apparently to an inability to couple the dehydrogenase and the diaphorase reactions. Similar considerations may be made in the case of TPN-linked isocitric dehydrogenase which also presented little activity in the absence of PMS but strong activity in the presence of this substance. It is therefore suggested that the dehydrogenase-diaphorase reactions only demonstrate the dehydrogenase fractions, which on a subcellular level are coupled to diaphorase and presumably *in vivo* are concerned with electron transfer to the

respiratory chain. This specificity of the dehydrogenase-diaphorase reactions seems surprising since reduced coenzyme generated by the non-diaphorase-coupled fractions might be expected to diffuse to the sites of diaphorase activity but apparently such diffusion is insufficient to produce a visible diaphorase reaction.

The dehydrogenase-diaphorase reactions demonstrated great activity of DPN-linked lactic, malic and isocitric dehydrogenase, particularly in the macrophages. This staining reaction, however is not seen after the addition of PMS to the media and in its place a variable staining is produced revealing a completely different activity pattern of the same enzymes. The dehydrogenase PMS reactions therefore seem to demonstrate selectively the non-diaphorase-coupled dehydrogenase fractions. The diaphorase-coupled DPN-linked enzymes are presumably mainly localised in the mitochondria, and the low permeability of the mitochondrial membrane to PMS (13) may explain that this substance does not replace diaphorase as intermediate electron carrier but it is uncertain why the diaphorase reaction is inhibited.

The dehydrogenase-PMS reactions give variable results in contrast to the constant results given by the dehydrogenase-diaphorase reactions. Probably this variability is due to loss of non-diaphorase-coupled dehydrogenases by diffusion during incubation, as also indicated by the occurrence of formazan precipitation in the incubation media (15) Such a difference between the solubility of the dehydrogenase fractions demonstrated by the two types of reactions may reflect differences in their intracellular localisation. The non-diaphorase-coupled dehydrogenases are probably mainly associated with glycolysis and reductive synthesis in the ground substance of the cytoplasm, whereas the diaphorase-coupled enzymes are confined to the mitochondria and—particularly in the case of glucose-6-phosphate dehydrogenase—to other organelles (17)

It remains to discuss the metabolic implications of the results. The high degree of glucose-6-phosphate dehydrogenase activity in all the inflammatory cells is an indication of glucose breakdown through the pentose shunt. Activity of this pathway in leukocytes has been demonstrated biochemically (1) The observation that by far the greater fraction of this enzyme is non-diaphorase coupled is consistent with the recent detection of an enzyme system in polymorphonuclears (from guinea pigs) capable of oxidizing TPNH in the presence of oxygen with the formation of

hydrogen peroxide (6) Otherwise TPNH may be oxidised by reductive TPN linked reactions.

DPN linked isocitric dehydrogenase is predominantly diaphorase-coupled particularly in macrophages, whereas the TPN linked form is chiefly non-diaphorase-coupled in both cell types. The diaphorase-coupled DPN linked isocitric dehydrogenase presents the same activity pattern as succinic dehydrogenase (16) and diaphorase-coupled DPN-linked malic dehydrogenase, all of which are part of the tricarboxylic acid cycle, which therefore seems to play a greater role in macrophages than in neutrophils. The function of non-diaphorase-coupled TPN linked isocitric dehydrogenase is less certain but it may be concerned with the generation of TPNH for reductive synthesis of lipids. These findings are in agreement with biochemical studies of other tissues. ERNSTER (4) first described the presence of a DPN linked isocitric dehydrogenase in rat liver This enzyme belonged to the mitochondrial fraction whereas the TPN linked form was mainly extramitochondrial. GOEBELL AND KLINGENBERG (5) found a constant ratio between the activity of DPN linked isocitric and malic dehydrogenase and the content of cytochrome *a* in mitochondria from different tissues, and concluded that these enzymes were coupled to the respiratory chain. Activity of TPN linked isocitric dehydrogenase however was unrelated to the cytochrome content.

Non-diaphorase-coupled lactic dehydrogenase presents greater activity than the other DPN linked enzymes. To judge from the most strongly stained preparations—which presumably offer the most correct picture—the activity of the non-diaphorase-coupled fraction in the neutrophils is considerably greater than that of the diaphorase-coupled fraction This result is consistent with the predominantly glycolytic metabolism of this cell type. The presence of diaphorase-coupled lactic dehydrogenase indicates that leukocytes may also utilise lactic acid, as already suggested by the experiments of SBARRA AND KARNOVSKY (12) The non-diaphorase-coupled DPN linked malic dehydrogenase probably corresponds to the supernatant malic dehydrogenase demonstrated in homogenisation studies of other tissues (3) whereas the weak TPN-linked malic dehydrogenase activity observed with both reactions may only reflect a small affinity of DPN-linked malic dehydrogenase to TPN

It is obvious that further studies are required to elucidate the effect of PMS on histochemical dehydrogenase reactions. Neverthe less it is hoped that the techniques of this study may prove of value for investigating leukocyte metabolism under pathological con ditions.

Summary

The activity of coenzyme-linked dehydrogenases in exudate leukocytes obtained by the 'skin-window' technique was examined histochemically using phenazine methosulphate (PMS) as intermediate electron carrier instead of indigenous diaphorase. The results differed greatly from those previously obtained using standard histochemical procedures, and it was concluded that the dehydrogenase PMS reactions demonstrated the non-diaphorase-coupled dehydrogenase fractions, whereas the standard reactions only revealed the dehydrogenase fractions, which were coupled to the respiratory chain. It was particularly noticeable that the exudate cells presented great activity of non-diaphorase-coupled TPN-linked glucose-6-phosphate and isocitric dehydrogenase, which had been left undetected using the standard reactions.

Résumé

Etude histochimique de l'activité des hydrogénases, liées aux coenzymes, de leucocytes de l'exsudat, obtenus par la méthode de la fenêtre cutanée. La métho-sulphate de phénazine (PMS) est employé à la place de la diaphorase endogène pour intermédiaire des électrons. Les résultats obtenus diffèrent fortement des résultats antérieurs, obtenus par la méthode histochimique usuelle. L'auteur conclut que les réactions déhydrogénases – PMS mettent en évidence les fractions des déhydrogénases, qui ne sont pas rattachés à la diaphorase, alors que les réactions standard déterminent seulement les fractions liées à la chaîne respiratoire. En outre on peut remarquer que les cellules de l'exsudat présentent une forte activité des déhydrogénases, liées au TPN du glucose-6-phosphate et de l'acide isocitrique, qui était pas révélée par la méthode usuelle.

Zusammenfassung

Die Aktivität von Koenzym-abhängigen Dehydrogenasen in Exsudat-Leukozyten, die mit der «Hautfenster»-Methode gewonnen worden, wurde histochemisch untersucht. Phenazinmethosulfat (PMS) wurde an Stelle der Diaphorasen der Zellen als intermediärer Elektronenträger verwendet. Die Ergebnisse zeigten starke Abweichungen von denjenigen, die früher mit histochemischen Standardmethoden erhalten worden waren. Daraus wird der Schluß gezogen, daß die Dehydrogenase-PMS-Reaktionen, die nicht an Diaphorase gebundenen Dehydrogenasefraktionen nachweisen, während die Standardreaktionen nur die an die Atmungskette gekoppelten Dehydrogenasefraktionen darstellen. Es fiel vor allem auf, daß die Exsudatzellen eine starke Aktivität von nicht an Diaphorase gekoppelter TPN-gebundener Glukose-6-phosphat- und Isozitronensäure-Dehydrogenase aufweisen, die mit den Standardreaktionen nicht nachgewiesen werden.

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Acta haemat. 32, 27–34 (1964)

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Die Heparinbehandlung bei erworbener hämolytischer Anämie

Von K. M. HEINE, H. HERRMANN UND H. STOBBE

Unter den immunhämatologischen Erkrankungen stellen die antikörperbedingten hämolytischen Anämien die Erkrankungsgruppe dar, über deren Pathogenese, Diagnostik, klinischen Verlauf und Behandlung wir am besten unterrichtet sind. Dennoch werden erworbene hämolytische Anämien leicht verkannt und ihre Behandlung wird nicht immer mit der notwendigen Konsequenz durchgeführt. Die Kranken kommen infolge des sehr wechselhaften und gelegentlich stürmischen Verlaufs dadurch leicht in lebensbedrohliche Situationen. Die Erfolgschancen der verspätet einsetzenden Therapie sind dann erheblich gemindert.

Als Standardtherapie der antikörperbedingten hämolytischen Anämien kann heute eine anfangs hoch dosierte Corticoidbehandlung mit Übergang auf eine Erhaltungsdosis gelten, die meist gute Erfolge hat. Die Splenektomie sollte erst dann in Erwägung gezogen werden, wenn einer sachgemäß durchgeführten Corticoidtherapie ein Erfolg versagt bleibt und nachgewiesen wurde, daß die Milz auch zahlenmäßig den stärksten Erythrocytenabbau bewirkt. Durch Cr^{51}-Markierung der Erythrocyten und Lienoscintigraphie kann die Abbaufunktion der Milz am sichersten beurteilt werden.

Bei Anämien, die durch komplette Antikörper hervorgerufen werden und bei denen der Erythrocytenuntergang in stärkerem Maße in der freien Blutbahn erfolgt, hat sich eine Corticoidtherapie als nicht wirkungsvoll erwiesen.

Diese Tatsache ist bedeutungsvoll, weil schwere hämolytische Krisen oft mit einem starken Anstieg der Kälteantikörper einhergehen. Von Corticoidgaben ist dann nur ein verzögert eintretender Wirkungseffekt zu erwarten.

Bei einem großen Teil der antikörperbedingten Anämien sind die Behandlungserfolge durchaus günstig. Die Spätprognose dieser Erkrankungen bleibt jedoch nach GRABAR und EYQUEM (4) noch zweifelhaft. Größere therapeutische Schwierigkeiten bestehen bei schweren hämolytischen Schüben oder bei unsachgemäß vorbehandelten Patienten. Nach Beginn einer ausreichend hoch dosierten Corticoidbehandlung kommt es meist nicht mehr zu einem weiteren Absinken der Erythrocytenzahl, eine deutliche Besserung des Blutbildes und des Gesamtzustandes kann sich aber entsprechend der Halbwertszeit der Antikörper erst nach mehreren Tagen anbahnen.

Owren (8) versuchte 1949 erstmals hämolytische Anämien mit Heparin therapeutisch zu beeinflussen Owren erkannte den schnellen therapeutischen Effekt, der später von Storti et al. (13) Roth und Frumin (11) McFarland et al. (7) sowie von Wagner et al. (14) bestätigt wurde. Auch wir möchten den besonderen Wert der Heparintherapie erworbener hämolytischer Anämien in dieser Besonderheit sehen. Allerdings ist die sofortige Wiederverschlechte rung des klinischen Bildes nach Absetzen des Heparins ebenso charakteristisch wie der schnelle Wirkungseintritt. Über ein Ver sagen der Heparinbehandlung bei einem Patienten berichten in dem uns bekannten Schrifttum nur Böhnel und Stacher (1)

Wir sehen eine Indikation zur Heparinbehandlung der erwor benen hämolytischen Anämie 1 bei hämolytischen Krisen, 2. bei unverzüglich behandlungsbedürftigen Patienten, die sich wegen verspäteter Einweisung oder Nichterkennung in einem bedrohli-chen Zustand befinden und 3 bei einem Versagen der üblichen Therapie. Die Kontraindikationen einer Antikoagulantientherapie sollen in diesem Zusammenhang nicht erörtert werden. Im Folgen-den wird über zwei Patienten berichtet, bei denen eine Kurzzeit bzw eine Langzeitbehandlung mit Heparin durchgeführt wurde.

Kasuistik

1 Patientin K A., geb. 13. Februar 1938, Laborantin. Die Anamnese bietet bis zum 20. Lebensjahr abgesehen von häufigen Kopfschmerzen keine Besonderheiten. 1958 sei vor der Geburt ihres ersten Kindes erstmals eine Blutarmut aufgetreten. Nach dem Bericht einer auswärtigen Klinik wurde im Februar 1959 eine infizierte stiel-gedrehte Dermoidzyste entfernt. Histologisch fanden sich keine Hinweise auf maligne Entartung Bereits vor der Operation wurde bei der Patientin eine Anämie festgestellt (44% Hämoglobin nach Sahli) Die Blutkörperchensenkungsgeschwindigkeit war damals mit 136 mm in der ersten Stunde und 140 mm in der zweiten Stunde maximal beschleunigt. Eine Bluttransfusion wurde komplikationslos vertragen. Nach der Opera-tion habe sich die Patientin gut erholt. Vor ihrer zweiten Entbindung im Jahre 1960 sei es jedoch wiederum zu einer Blutarmut gekommen. Das gleiche habe sich im Januar Februar März und August 1962 wiederholt. Die Hämoglobinwerte seien damals innerhalb kurzer Zeit bis minimal 28% nach Sahli abgesunken. Die Behandlung der verschiedenen Anämieschübe sei ambulant mit Eisen- Kobalt-, Vitamin B_{12} und Folsäurepräparaten erfolgt. Noch 10 Tage vor der stationären Aufnahme wurde der Patientin wegen einer mäßigen Anämie von 60% Hämoglobin nach Sahli ein Folsäure-Vitamin-B_{12}-Kombinationspräparat verabfolgt. Es kam jedoch zu keiner Besserung, sondern zu verstärkten Allgemeinbeschwerden. Insbesondere traten Appetitlosigkeit, Schwäche und starke Kopfschmerzen auf. In diesem Zustand wurde uns die Patientin überwiesen.

Bei der Klinikaufnahme am 4. April 1963 befand sich die Patientin in stark reduziertem Allgemeinzustand. Auffallend waren extreme Blässe, subikterische Ver färbungen der Haut und deutlicher Sklerenikterus. Die Zunge war belegt, und es

bestand ein foetor ex ore. Über allen Herzostien hörte man ein systolisches Geräusch. Die Herzaktion war tachycard. Der Blutdruck betrug 120/80 mm Hg. Die Milz war zwei Querfinger unter dem Rippenbogen tastbar und von praller Konsistenz. Die Leber war nicht vergrößert.

Wesentliche Laboratoriumsbefunde bei der Klinikaufnahme: Blutkörperchensenkung nach der ersten Stunde 163 mm, nach der zweiten Stunde 167 mm. Hämoglobin 2,6 g% (Hämoglobincyanidmethode), Erythrocyten 670000/µl, Retikulocyten 529000/µl, Leukocyten 5900/µl, Thrombocyten 230000/µl. Differentialblutbild: Basophile 2%, Eosinophile 1,5%, neutrophile Stabkernige 3%, neutrophile Segmentkernige 65%, Lymphocyten 25%, Monocyten 2,5%, Lymphoidzellen 1%. Auf 100 Leukocyten fanden sich 3 Erythroblasten. Entsprechend dem großen Retikulocytenanteil lagen vorwiegend Makrocyten vor. Aber auch Ovalocyten und kugelförmige Erythrocyten waren in geringer Zahl nachweisbar. Es bestand eine Polychromasie.

Knochenmarksuntersuchung: Stärkste Hyperplasie der Erythropoese und Vermehrung der Retikulumzellen.

Serumeisen 141 γ% (Methode nach LAURELL). Bilirubin im Serum 2,16 mg%, davon 95% indirektes Bilirubin. Fibrinogen 251 mg% (Hitzefibrinmethode nach F. H. SCHULZ). Gesamteiweiß im Serum 6,5 g% (Biuretmethode). Papierelektrophorese des Serums: Albumin 54,4%, Alpha-1-Globulin 7,8%, Alpha-2-Globulin 8,1%, Beta-Globulin 12,0%, Gamma-Globulin 17,7%. Bromlaugen-Stickstoff 32 mg% (Methode nach KOWARSKI). Urin: Kein Nachweis von Proteinen, Zucker, Urobilin oder Bilirubin. Keine Vermehrung von Urobilinogen. Im Sediment Urate, Harnsäurekristalle und Plattenepithelien. Gerinnungsstatus: Quick-Wert unter 5%, Faktor II 42%, Faktor V 80%, Faktor VII 50%. Das Heparinantithrombin war extrem erhöht (über 2 Minuten). Das Serumantithrombin war dagegen normal. Die Gerinnungszeit war verlängert (Beginn nach 3 Minuten 30 Sekunden, Ende nach 9 Minuten 10 Sekunden).

Serologische Untersuchungen: Direkter Coombstest bei einem Titer von 1:32000 positiv, indirekter Coombstest bei einem Titer von 1:256 positiv. Vermehrung der Kälteagglutinine bis zu einem Titer von 1:1000, Verbreiterung der Wärmeamplitude, Autoagglutination. Inkompletter Kälteantikörpertiter 1:2 Millionen. Komplementwert 10 E/ml.

Auf Grund des klinischen Bildes und der Untersuchungsbefunde konnte die Diagnose einer antikörperbedingten hämolytischen Anämie gestellt werden. Die Behandlung wurde mit 120 mg Prednison eingeleitet (Abb. 1). Unter dieser Therapie war kein weiteres Absinken der Erythrocytenzahl zu beobachten, jedoch blieben die Zeichen einer schweren Hypoxämie unverändert bestehen. Es kam zum partiellen Nierenversagen mit Anstieg des Rest-Stickstoff auf 79,5 mg%. Die Patientin klagte über starke Kopfschmerzen und litt unter unstillbarem Erbrechen. Ein geringer Anstieg des *Hämoglobins* auf 5,2 g% ist am ehesten auf die Exsiccose infolge des Erbrechens zurückzuführen. Von Bluttransfusionen mußte wegen der starken Agglutinationsbereitschaft Abstand genommen werden. In diesem äußerst bedrohlichen Zustand wurde unter Fortführung der Prednisontherapie die Heparinbehandlung begonnen. Wir gaben der Patientin zweimal 15000 IE (2mal 150 mg) Heparin i.v. in einstündigem Abstand. Die weitere Behandlung erfolgte mit täglich 50000 IE (500 mg) eines Depot-Heparinpräparates i. m. Der Erfolg war eindrucksvoll. Bereits wenige Minuten nach der ersten Injektion sistierte das Erbrechen und die Kopfschmerzen verschwanden. Die Patientin fühlte sich trotz der weiterbestehenden Rest-Stickstofferhöhung von 117,6 mg% wohl. Im weiteren Verlauf kam es zu einem kontinuierlichen Hämoglobinanstieg und Titerabfall der inkompletten Wärmeantikörper und Kälteagglutinine. Wir führten die Heparinbehandlung über 20 Tage durch. Die Prednisontherapie wurde als Dauerbehandlung fortgesetzt. Nach Absetzen des Heparins trat kein nachweisbarer hämolytischer Schub ein. Der weitere Krankheitsverlauf zeigte keine Besonderheiten.

Hb g%

Heparin

Prednison

Abb. 1 Kurzzeit-Heparinbehandlung bei antikörperbedingter hämolytischer Anämie.

2. *Patientin P. E.*, geb. 30. August 1910, ungelernte Arbeiterin. Anamnese: Bis zu ihrem 35. Lebensjahr habe die Patientin mehrmals Anginen durchgemacht. Eine Gelbsucht sei erstmals im Frühjahr 1961 aufgetreten, worauf in einer auswärtigen Klinik die Diagnose einer erworbenen hämolytischen Anämie gestellt wurde. Aus dem Krankenblatt geht hervor, daß es im Januar, März und August 1962 erneut zu hämolytischen Schüben gekommen ist, die eine stationäre Aufnahme erforderlich machten. Die offensichtlich unterdosierte Prednisontherapie (maximal 30 mg/die) führte zu keinem dauerhaften Erfolg. Da ein deutlicher Milztumor bestand, wurde die Splenek-

tomie durchgeführt und gleichzeitig die Corticoidbehandlung beendet. Der Erfolg der Milzexstirpation war jedoch nur vorübergehend. Schon nach drei Monaten sei es erneut zu Mattigkeit, Gewichtsabnahme und nach einem Schüttelfrost zu dunkler Verfärbung des Urins gekommen. Es wurden nochmals Corticoide verabfolgt. Da keine Besserung erzielt werden konnte, wurde die Patientin zu uns überwiesen.

Bei der Klinikaufnahme am 7 Januar 1963 befand sich die Patientin in leicht reduziertem Allgemeinzustand. Die Haut und die sichtbaren Schleimhäute waren schlecht durchblutet. Es bestand kein Ikterus. Die Tonsillen waren deutlich vergrößert und zerklüftet, jedoch ohne Zeichen einer akuten Entzündung. Das Gebiß wies mehrere schadhafte Zähne auf. Der Leberrand war zwei Querfinger unterhalb des Rippenbogens tastbar. Im linken Oberbauch fand sich eine reizlose Operationsnarbe nach Splenektomie. Röntgenologisch konnte eine Cholelithiasis nachgewiesen werden.

Wesentliche Laboratoriumsbefunde bei der Klinikaufnahme Blutkörperchensenkung nach der ersten Stunde 92 mm, nach der zweiten Stunde 100 mm. Hämoglobin 11,3 g% (Hämoglobincyanidmethode) Erythrocyten 3 11 Millionen/µl, Retikulocyten 576000/µl, Leukocyten 10100/µl, Thrombocyten 503000/µl. Differentialblutbild Basophile 2%, Eosinophile 2%, neutrophile Stabkernige 2%, neutrophile Segmentkernige 68% Lymphocyten 22% Monocyten 4%. Es bestand eine Anisocytose, vereinzelt fanden sich Ovalocyten.

Knochenmarkuntersuchung Starke Hyperplasie der Erythropoese, Vermehrung der Plasmazellen.

Serumeisen 72 γ% (Methode nach LAURELL) Bilirubin im Serum 0,87 mg% Fibrinogen 245 mg% (Hitzefibrinmethode nach F H. SCHULZ) Gesamteiweiß im Serum 6,65 g% (Biuretmethode) Papierelektrophorese des Serums Albumin 46,0%, Alpha-1-Globulin 7,2%, Alpha-2-Globulin 6,1% Beta-Globulin 16,8% Gamma-Globulin 23,9%

Urin Kein Nachweis von Proteinen, Zucker Urobilin oder Bilirubin. Keine Vermehrung von Urobilinogen. Im Sediment vereinzelt Leukocyten und Plattenepithelien.

Serologische Untersuchungen Direkter Coombstest bei einem Titer von 1 256 positiv Indirekter Coombstest negativ Keine Vermehrung der Kälteagglutinine, keine Autoagglutination.

Die Untersuchungsbefunde bestätigten die Diagnose einer antikörperbedingten hämolytischen Anämie. Obwohl bereits aus der Anamnese eine schlechte Ansprechbarkeit auf Corticoide bekannt war leiteten wir nochmals eine Prednisonbehandlung in hoher Dosierung ein (Abb. 2) Unter dieser Therapie kam es jedoch mehrfach zu hämolytischen Schüben leichteren Grades (30. Januar 1963, 26. Februar 1963, 26. März 1963) mit Absinken der Hämoglobinwerte bis minimal 7 7 g% ohne faßbare Erhöhung des Bilirubinspiegels. Es fiel auf, daß dem Hämolyseschub jeweils eine mehr oder minder starke Erhöhung der Körpertemperatur und Gliederschmerzen vorausgingen. Im Anschluß an eine Zahnextraktion, welche unter Prednison und antibiotischem Schutz durchgeführt wurde, entwickelte sich eine unspezifische Lymphadenitis colli mit Temperaturanstieg auf 38 bis 39 °C. Es kam zu einer schweren hämolytischen Krise. Die Hämoglobinwerte fielen auf 3 g% bei 710000 Erythrocyten/µl. Die Retikulocytenzahl betrug 500000/µl. Das Serumbilirubin stieg auf 4,08 mg% der Komplementwert lag bei 64 E/ml. Die Blutkörperchensenkungsgeschwindigkeit war mit 140 mm in der ersten und 143 mm in der zweiten Stunde maximal beschleunigt. Die Patientin wurde benommen und war zeitweilig nicht mehr ansprechbar. Eine Steigerung der Prednisondosis auf 150 mg konnte das hämolytische Geschehen nicht aufhalten. Nur durch vier Bluttransfusionen gelang es zunächst, den schweren Zustand zu überbrücken. Jedoch kam es zu einem raschen Ansteigen des Coombs Titers bis 1 2000 und der Kälteagglutinine bis 1 1000, so daß weitere Transfusionen nicht möglich waren. Da die Erythrocytenwerte erneut absanken, entschlossen wir uns zur Heparin-

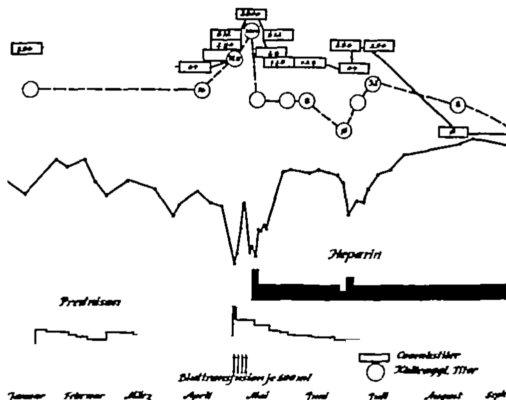


Abb. 2. Langzeit-Heparinbehandlung bei antikörperbedingter hämolytischer Anämie mit schlechter Ansprechbarkeit auf Prednison und Zustand nach Splenektomie.

behandlung. Wir begannen diese mit 50 000 IE (500 mg) und gingen später auf 25 000 IE (250 mg) Depot Heparin i.m. zurück. Der weitere Verlauf zeigte eine Normalisierung des roten Blutbildes und einen raschen Abfall der Autoantikörper. Als versucht wurde, die Dosis weiter zu reduzieren, trat erneut ein leichter hämolytischer Schub ein, der durch Steigerung der Heparinmenge gut beherrscht werden konnte. Da bei der Patientin offenbar eine verminderte Ansprechbarkeit auf Prednison vorliegt, leiteten wir eine Heparindauerbehandlung ein. Diese wird bereits über 6 Monate durchgeführt. Die Patientin ist seit 3½ Monaten aus der stationären Behandlung entlassen. Sie führt die intramuskulären Injektionen mit zweimal 12 500 IE (125 mg) täglich selbst durch und wird wöchentlich einmal in unserer hämatologischen Ambulanz kontrolliert. Abgesehen von mäßigem Haarausfall und kleineren Hämatomen an den Injektionsstellen beobachteten wir keine Nebenerscheinungen, insbesondere keine allergischen Symptome.

Der Wirkungseffekt des Heparins ist in beiden geschilderten Fällen eindeutig, auch wenn gleichzeitig eine Prednisonbehandlung durchgeführt wurde. Zweifellos stellen die täglich notwendigen intramuskulären Injektionen für die Patientin eine Belastung dar. Eine solche Therapie stellt außerdem an die Intelligenz und Ein

seitens der Patienten gewisse Anforderungen. Nicht immer sind diese Voraussetzungen gegeben. Auch wissen wir wenig über die Auswirkungen einer langfristigen Heparinverabreichung; allergische Komplikationen wurden wiederholt beschrieben (2, 5, 9, 10, 14) und vor einer Langzeitbehandlung gewarnt. Es bleibt abzuwarten, ob Indandion- und Cumarinpräparate bei der Dauerbehandlung hämolytischer Anämien das Heparin ersetzen können (3).

Über den Wirkungsmechanismus des Heparins, der zu einem schnellen Sistieren des beschleunigten Erythrozytenabbaus führt, ist wenig Sicheres bekannt. Es wird neben einer Antithrombin-, eine Antikomplementwirkung (13), ein Antilysolecithinase-Effekt (12, 13) sowie ein Ladungseffekt des Heparins (6, 14) diskutiert. Bei konstitutionellen hämolytischen Anämien kann durch Heparin keine Besserung erzielt werden (13, 14). Zweifellos ist der Angriffsort der Heparinwirkung am Erythrozyten selbst zu suchen. Die Antikörperproduktion scheint nicht beeinflußt zu werden. Eigene Untersuchungen ergaben, daß der Kontakt von Heparin mit den Erythrozyten weder in vitro noch in vivo zu einer Verminderung der Antikörperbeladung führt. Auch Storti und Vaccari (13) fanden in ihren Experimenten keinen Einfluß von Antikoagulantien auf die Antikörperbindung. Für die verschiedenen Deutungsversuche der Heparinwirkung lassen sich Beweise und Gegenbeweise erbringen. Wahrscheinlich handelt es sich um ein recht komplexes Geschehen. Bemerkenswert erscheint die Antikomplement- und Antilysolecithinase-Wirkung des Heparins, da sowohl Komplement als auch Lysolecithinase beim Erythrozytenabbau eine ausschlaggebende Rolle spielen.

Abschließend läßt sich gegenwärtig sagen, daß das Heparin bei der Behandlung erworbener hämolytischer Anämien in besonders gelagerten Fällen durchaus eine Berechtigung besitzt. Der Wert liegt in dem sofortigen Wirkungseintritt, über dessen Zustandekommen wir allerdings noch nicht genügend unterrichtet sind.

Zusammenfassung

Es wird über die Heparinbehandlung antikörperbedingter hämolytischer Anämien berichtet. Eine Indikation ist gegeben 1. wenn ein schneller Wirkungseffekt erforderlich ist, wie zum Beispiel in hämolytischen Krisen und 2. bei Versagen der Corticoid-behandlung und Splenektomie. Für beide Indikationsstellungen wird das unterschiedliche therapeutische Vorgehen als Kurzzeit- bzw. Langzeit-Heparinbehandlung an Hand von zwei charakteristischen Krankheitsverläufen erläutert. Der Wirkungsmechanismus, sowie die Gefahren einer Dauertherapie werden kurz erörtert.

Summary

Heparin therapy of haemolytic anaemia due to antibodies is reported. This treatment is indicated 1) when a rapid effect is essential, and 2) when corticotherapy and splenectomy has been of no avail. The different modes of treatment for the two indications, as brief and prolonged heparin therapy respectively are described on the basis of two typical cases. The mechanism of action and the dangers of long-term treatment are briefly discussed.

Résumé

Rapport sur le traitement d'anémies hémolytiques immunologiques par l'héparine. L'indication de ce traitement est donné 1 lorsqu'un effet rapide est nécessaire, par exemple pendant les crises hémolytiques, et 2. après un échec du traitement cortisonique et de la splénectomie. Pour les deux indications le procédé thérapeutique différent d'un traitement héparinique à court et à long terme est illustré par deux évolutions caractéristiques de la maladie. Le mécanisme ainsi que les dangers du traitement sont brièvement discutés.

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Acta haemat. 32: 35–38 (1964)

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Binding of Bilirubin from Red Cell with Glucose-6 Phosphate-Dehydrogenase Deficiency*

By Francesco Schettini and Tullio Meloni

The capacity of erythrocytes to binding the unconjugated form of bilirubin was demonstrated by Watson (1) Oski and Naiman (2) This binding ability appears related to the physiochemical property of the red cell membrane and particulary of their lipids. The binding of bilirubin is impaired in erythrocytes coated with incomplete antibodies (2)

We have investigated the ability of the red cell with glucose 6-phosphate-dehydrogenase (G6P DH) deficiency to absorb the unconjugated bilirubin.

Material and Methods

Serum rich in unconjugated bilirubin was obtained from 3 jaundiced newborn children with erythrocyte-enzyme deficiency (G6P—DH) and from premature baby

Compatible red cells were obtained from 5 G6P—DH deficient Sardinian subjects and from 6 normal subjects with the use of ACD as anticoagulant.

Red cell were stored at 4 °C and tested at intervals ranging from 12 hours to three days. Red cells were washed once with isotonic saline before use.

Determinations of unconjugated bilirubin were performed in duplicate with the method of Malloy and Evelyn (3) and with the spectrophotometric method of Eberlein (4).

The experiments of binding were carried out according to the method of Oski and Naiman (2).

The activity of G6P—DH of red blood cells was tested according to Zinkham et al. (5)

A Coombs test as performed on the mixture at the end of incubation.

The results were expressed as terms of mg% of bilirubin binding per packed cell volume of 50% and as % of bilirubin reduction per packed cell volume of 50%

Investigation supported by research grant from C.N.R., Italy

Table 1

Number	Sex	G6P DH activity of erythrocytes, U/100 ml of red blood cells	Evelyn Method: Bilirubin reduction per packed cell volume of 50%, mg%			Evelyn Method: % reduction of initial bilirubin level per packed cell volume of 50%			Malloy and Evelyn Method: Bilirubin reduction per packed cell volume of 50%, mg%			Malloy and Evelyn Method: % reduction of initial bilirubin level per packed cell volume of 50%		
			0	60 min	total	0	60 min	total	0	60 min	total	0	60 min	total
1	F	120	0.50	0.40	0.90	7.8	6.2	14.0	0	0.95	0.95	0	14.5	14.5
2	M	233	0	0.15	0.15	1.12	1.12	2.24	0.45	1.33	1.78	3.7	11.0	14.7
3	M	152	1.74	1.43	3.17	12.2	10.0	22.2	0.14	2.22	2.36	1.16	18.5	19.6
4	M	145	3.92	0	3.92	21.7	0	21.7	0.80	2.58	3.38	5.0	16.1	21.1
5	M	202	2.21	0.85	3.06	12.2	4.72	16.9	1.25	0	1.25	7.81	0	7.81
6	M	130	1.35	0	1.35	8.01	0	8.01	2.35	1.21	3.56	14.6	7.56	22.16
		Mean value			2.09			14.17			2.21			16.61
					±2.30						±2.41			
7	M	0	1.95	0	1.93	26.2	0	26.2	0	0	0	0	0	0
8	M	0	0	0.62	0.62	0	4.62	4.62	2.14	0	2.14	17.8	0	17.8
9	M	13	2.62	0.21	2.83	18.4	1.41	19.8	1.89	1.38	3.27	15.7	11.5	27.2
10	M	0	1.45	0.78	2.23	8.68	4.67	13.3	2.20	2.00	4.20	13.7	12.5	26.2
11	M	19	1.41	0	1.41	8.44	0	8.44	2.42	0	2.42	15.1	7.31	22.4
		Mean value			1.80			14.48			2.40			18.72
		σ			±1.49						±2.18			
P = 0.05		t			0.221						0.285			
					0.437						0.109			

Results and Discussion

Erythrocytes of normal subjects and with G6P DH deficiency show a consistent ability to absorb small amounts of bilirubin. The mean value of reduction employing the bilirubin method of Eberlein was of 2.09 mg% per packed cell volume of 50% for normal erythrocytes and of 1.80 mg% for erythrocytes with G6P DH deficiency. Per cent of bilirubin reduction was of 14.17 and respectively of 14.48. The mean value of reduction with the bilirubin method of Malloy and Evelyn was of 2.21 mg% per packed cell volume of 50% for normal erythrocytes and of 2.40 mg% for erythrocytes with G6P DH deficiency. Per cent of bilirubin reduction was of 16.64 and respectively of 18.72 (table I).

The difference of binding of bilirubin between red cell with normal and deficient G6P DH activity was not significant (t = 0.221 and 0.285; x = 0.457 and 0.103; P = 0.05).

Erythrocytes with G6P DH deficiency are capable of binding bilirubin as normal red cells. The reduction of total lipids in G6P DH deficient erythrocytes (6) and the relative abnormality in the lipoprotein membrane, however, does not change the capacity of these red cells to absorb the unconjugated form of bilirubin.

Summary

The binding capacity for bilirubin of erythrocytes with G6P—DH deficiency evaluated by incubation with serum rich in unconjugated bilirubin, is likely to the one of red cell with normal G6P—DH activity.

Résumé

Détermination de la capacité di fixation de la bilirubine des érythrocytes déficitaires de la G6P–DH par incubation dans le sérum riche en bilirubine non-conjugée. La capacité des erythrocytes déficitaires est semblable à celle des érythrocytes avec une activité normale de la G6P–DH.

Zusammenfassung

Durch Inkubation mit Serum, das reich an unkonjugiertem Bilirubin war, wurde die Bindungsfähigkeit von roten Blutkörperchen für Bilirubin bestimmt. Sie war bei Erythrocyten mit einem Mangel an G6P–DH ähnlich wie bei Erythrocyten mit normaler G6P–DH-Aktivität.

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Acta haemat. 32: 39–43 (1964)

Aus der medizinischen Abteilung des Kaiserin-Elisabeth-Spitales der Stadt Wien
(Vorstand: Prof. Dr. R. Klima)

Vorkommen und Bedeutung von Endothelzellen im peripheren Blut*

Von E. Gött

Im Leukozytenkonzentrat, wie es von Klima et al. vor etwa 15 Jahren angegeben wurde (1) und seither routinemäßig in der klinischen Hämatologie angewendet wird, kommen neben anderen seltenen Zellen immer wieder auch Endothelzellen bzw. deren Kerne zur Beobachtung. Wir haben nun seit vier Jahren diese Zellen registriert, um ihre Bedeutung für die Klinik zu erfassen und berichten im folgenden darüber.

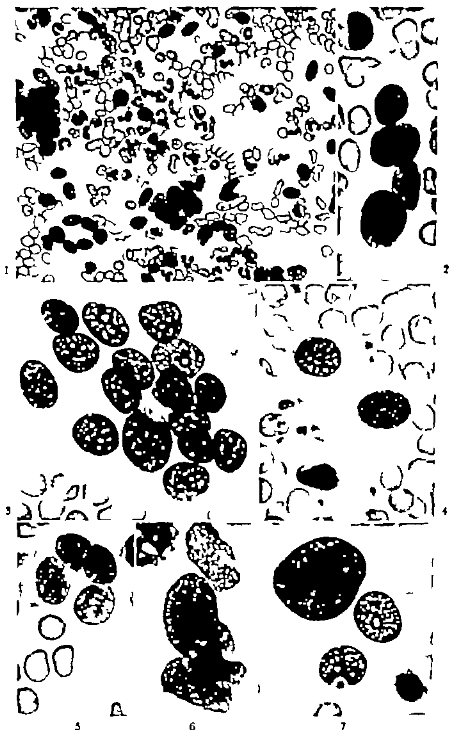
Methodik und Material

Über die Methodik des Leukozytenkonzentrates haben wir bereits oftmals ausführlich berichtet (zuletzt 2), so daß wir uns hier kurz fassen können. Es werden 5 ml Venenblut mit 0,4 ml Natr. citric. (3,8%ig) gemischt und nach schonender Zentrifugierung die meist gut erkennbare Leukozytenschicht abgehoben, ausgestrichen und dem Bedarf entsprechend gefärbt. Die Untersuchungen wurden durchgeführt an einem nicht ausgewählten klinischen Material.

Ergebnisse

Wir haben im Zeitraum vom September 1959 bis Mai 1963 das sind 3 Jahre und 8 Monate, insgesamt 5738 Leukozytenkonzentrate untersucht. Darunter fanden sich in 50 Fällen Endothelzellen, was einem Prozentsatz von 0,84 entspricht. Ihr Vorkommen kann jedoch in rund 1% der untersuchten Fälle angenommen werden und zwar aus folgenden Gründen. Wir haben zu Anfang der Untersuchungen nicht mit der gleichen Aufmerksamkeit nach den Zellen gesucht wie jetzt, da wir ihre Häufigkeit unterschätzt haben; es können einige wenige Zellen übersehen werden und es werden auch

* Mit Unterstützung durch den wissenschaftlichen Fonds der Stadt Wien.


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nicht alle Zellen des Leukozytenkonzentrates ausgestrichen und begutachtet, so daß auch Endothelzellen zurückbleiben können schließlich liegt der Prozentsatz bei erhöhter Aufmerksamkeit in den letzten 1½ Jahren, das ist bis Ende Januar 1964 bereits über 1

Von den 50 Fällen mit Endothelzellen die hier besprochen werden waren 31 Frauen und 19 Männer was jedoch der allgemeinen Geschlechtsverteilung unseres Patientengutes entspricht der jüngste Patient war 12 Jahre, der älteste 90 Jahre, der Altersdurchschnitt betrug 63 Jahre. 33 Patienten (66°) sind als schwerkrank zu bezeichnen darunter sind 13 (26 /) die kurze Zeit vor ihrem Ableben standen In 17 Fällen (34 ,) war zum Zeitpunkt der Blutabnahme die Körpertemperatur über 37 C davon jedoch nur in 3 Fällen (6%) über 38 C.

Die Endothelzellen werden also bei erwachsenen Patienten beiderlei Geschlechts und in jedem Alter gefunden Die schwerkranken Patienten machen mehr als die Hälfte des Patientengutes aus, Gesunde finden sich nicht darunter Eine vermehrte Ausschwemmung bei fieberhaften Zuständen konnte nicht beobachtet werden.

Die Endothelzellen bzw ihre Kerne liegen in den Ausstrichen manchmal einzeln und dann meist nicht weit voneinander entfernt. Öfters sind sie zu verschieden großen Gruppen angehäuft (Abb 1 3) Das Zytoplasma ist hell basophil sehr zerfließlich und oft unscharf begrenzt (Abb 2 4 5) Es besteht eine gewisse morphologische Ähnlichkeit mit den Retikulumzellen eine Speicherung oder Phagozytose konnten wir jedoch, zum Unterschied von diesen, nicht sehen. Auch mehrkernige Zellen (Abb 5) bzw kleinere synzytiale Verbände können gefunden werden Nicht selten sind jedoch nur die Kerne der Endothelzellen ohne Plasma vorhanden (Abb 3) sie sind meist oval und besitzen eine durchschnittliche Größe von etwa 8 12 µ erreichen aber in einzelnen Exemplaren bis über 20 µ (Abb 6 7) Sie besitzen eine charakteristische grobe, retikuläre

Abb 1 Endothelzellen bzw deren Kerne in Gruppen reichliche Aussaat bei Miliartbc. der Lunge. 520

Abb 2 Endothelzellen mit Kerben Panmyelopathie × 1000.

Abb. 3 Gruppe von Endothelzellkernen frischer Cerebralinsult. 1000.

Abb 4 Endothelzellen mit Zytoplasma Myeloblastose. 1000.

Abb 5. Mehrkernige Endothelzelle Panmyelopathie 1000.

Abb 6 und 7 Abnorm große Endothelzellkerne Lupus erythematodes dissem. und Cerebralinsult. 1000.

Struktur und lassen in der Regel einen, manchmal auch zwei basophile Nukleolen erkennen. Nicht ganz selten weisen sie auch eine in der Längsrichtung verlaufende «Kerbe» auf (Abb. 2).

Die Zahl der Endothelzellen pro Objektträgerausstrich variiert von 5 bis maximal 172. Die höchsten Werte fanden sich bei Schwerkranken, wie bei zwei Fällen von Miliartuberkulose, bei ausgedehntem Herzinfarkt und frischem Cerebralinsult, bei hämorrhagischer Cystopyelonephritis, bei schwerer Hepatitis und bei Coma hepaticum im Verlauf einer Cirrhose, bei Panmyelopathie mit hohem Fieber, bei generalisierter Lymphogranulomatose, bei Retothelsarkom, Peritonealkarzinose und maligner Knochenmetastasierung. In einigen Fällen konnten die Zellen bei ein und demselben Patienten auch in mehreren, in verschiedenen Abständen abgenommenen Blutproben nachgewiesen werden, so z.B. in drei verschiedenen Leukozytenkonzentraten innerhalb von 12 Tagen bei einem Fall mit Überempfindlichkeitsreaktion auf Hydantoin und generalisierten Drüsenschwellungen. Interessant ist noch die Beobachtung, daß es bei diesen Kranken mit Endothelzellen im Blut gleichzeitig zu einer pathologischen Ausschwemmung verschiedener unreifer Zellelemente aus den blutbildenden Geweben kommt: es werden auch Myelozyten und Promyelozyten gefunden, vereinzelt auch Myeloblasten und Retikulumzellen, dann lymphatische Reaktionsformen und histiozytäre Plasmazellen sowie auch Megakaryozyten und Erythroblasten. Mitunter sind auch verschiedene Phagozytosen anzutreffen.

Diskussion

Es wurde bisher nur von wenigen Autoren auf das Vorkommen *von Endothelzellen im Blut hingewiesen (3, 4), doch liegt unseres* Wissens noch kein Bericht über die Häufigkeit der Zellen und ihre *Bedeutung vor. Aus unseren Untersuchungen geht hervor, daß die* Endothelzellen im Blut nicht selten anzutreffen sind und daß sie bei schweren Erkrankungen auch in größerer Anzahl vorkommen. Was ihre Herkunft anlangt, muß man in erster Linie eine Abschilferung von Gefäßendothelien bei Gefäßschädigung im Verlauf schwerer Krankheitsprozesse annehmen. Wie weit Endothelzellen aus anderen retikuloendothelialen Geweben stammen, kann nicht sicher beurteilt werden. In einem Fall von Retotheliose waren jedoch reichlich Endothelzellen vorhanden, was dafür spricht, daß sie auch aus anderen Geweben kommen könne. In einzelnen Fällen

kann vielleicht auch eine mechanische Einschwemmung von Endothelzellen als Folge des Einstiches bei der Blutabnahme sowie der vorausgehenden Anlegung einer Stauung vorkommen doch dürfte diese Möglichkeit nach dem eben Gesagten keine besondere Rolle spielen.

Eine weitere Bedeutung haben die Endothelzellen für den heute an vielen Stellen betriebenen Tumorzellennachweis im peripheren Blut, der meist mit der gleichen oder mit sehr ähnlichen Methoden durchgeführt wird. Bei Unkenntnis der Ausschwemmung und der Morphologie der Endothelzellen, besonders der nackten und in ihrer Größe stark schwankenden Zellkerne mit ihren Nukleolen (Abb 6 und 7) ist eine Verwechslung mit Tumorzellen durchaus naheliegend

Zusammenfassung

In etwa 1 ‰ der Fälle finden sich bei einem nicht ausgewählten klinischen Krankengut in den Altersstufen von 12 bis 90 Jahren und bei beiden Geschlechtern mit Hilfe des Leukozytenkonzentrates Endothelzellen bzw deren Kerne im peripheren Blut. Die Zellen wurden in 50 Fällen nachgewiesen und zwar besonders zahlreich bei Schwerkranken, in einigen Fällen sogar wiederholt. Auf die Möglichkeit der Verwechslung mit Tumorzellen wird aufmerksam gemacht.

Summary

In about 1 ‰ of an unselected hospital population of both sexes in the age range of 12–90 years, endothelial cells or their nuclei can be shown in the peripheral blood by means of leukocyte concentration. These cells were demonstrated in 50 patients, particularly frequently in the severely ill and in some cases on repeated occasions. Attention is drawn to the possible confusion with tumour cells.

Résumé

L'auteur trouve à l'aide de la concentration leucocytaire chez env 1% de cas cliniques hétérogènes agées de 12–90 ans et des deux sexes, des cellules endothéliales, ou leurs noyaux, dans le sang périphérique. La présence de ces cellules était démontrée chez 50 cas, particulièrement nombreuses chez des personnes gravement malades, même plusieurs fois dans quelques cas. L'attention est attirée sur les possibilités de confusion avec les cellules de tumeurs.

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Acta haemat. 12: 44–62 (1964)

From the Cancer Research Unit, Veterans Admin. Hospital Bronx, New York

How Many Different Viruses Causing Leukemia in Mice?

By Ludwik Gross

The term "Mouse Leukemia" refers to a limited variety of forms of this disease observed in mice to develop spontaneously or induced either by X-ray irradiation, by carcinogenic chemicals, or by hormones. The most frequently observed form is lymphatic.

This is true for the great majority of strains of mice as well as for the general mouse population. The usual prominent feature of this form of leukemia is a large thymic lymphosarcoma; there is often, but not always, a concomitant enlargement of spleen, and less frequently also that of the liver. In the fully developed disease there may be also present a prominent enlargement of axillary and inguinal, as well as mesenteric, lymph nodes. Microscopic sections of internal organs, such as spleen, liver, or kidneys, show infiltration with leukemic cells, particularly in animals with generalized leukemia; this is not always the case, however, since in some mice in early phases when the disease is limited to a thymic lymphosarcoma, there may not be any apparent infiltration of internal organs. The degree of maturation of the leukemic cells, of invasion of peripheral blood, and of infiltration of internal organs may vary. In certain instances, acute lymphatic or stem-cell leukemia can be observed. It is quite probable that all these pathological manifestations represent rather different phases of the same progressive disease, than distinct disease entities.

Generalized Lymphosarcoma, the Most Frequent Form of "Leukemia" in Mice

In the majority of mice that develop spontaneously leukemia, the peripheral blood morphology does not reflect the presence of this disease. In such animals the disease can actually be designated as lymphosarcoma. In some animals only isolated lymphosarcomas may be found either in the thymus, or less frequently, and particularly in older mice, in the mesenteric lymph nodes. In most instances, however, multiple lymphosarcomas can be observed, involving the thymus, spleen, liver and peripheral lymph nodes.



Fig. 1 Leukemia Induced with Passage A Virus or with the Moloney Virus Strain. These 2 mice, both of the BALB inbred line, were inoculated (0.2 to 0.3 ml, i.p. each) when less than 5 days old, with either passage A leukemic 10^ filtrate (mouse A) or with the Moloney virus strain 10^ filtrate (mouse M). Both mice developed generalized leukemia about 2½ months after inoculation. The macroscopic and microscopic morphology were the same in both mice. Note the very large spleens and livers, large thymic and mesenteric tumors, also enlarged peripheral lymph nodes. On microscopic examination of blood smears the form of leukemia was diagnosed as myeloid in both mice.

The term leukemia can be applied to this form of disease only in its broad and general meaning; it does not necessarily imply changes in peripheral blood morphology. Actually, the most commonly occurring form of leukemia in mice does not show leukemic manifestations in peripheral blood, and could be designated as a generalized lymphosarcoma. This form of lymphoid leukemia in mice is essentially similar to visceral lymphomatosis in the fowl, the most common form of the chicken leukosis complex (16).

Myeloid leukemia also occurs spontaneously, or can be induced in certain strains of mice by inoculation of the mouse leukemia virus (18) or by X-rays (21), but is considerably less frequent than the lymphatic form. In some instances it may be difficult to

recognize the myeloid form on the basis of the macroscopic picture alone. I certain instances, in mice (fig 1) as well as in rats, w hav seen moderately large thymic tumors even though such animals had myeloid leukemia. However more often in myeloid leukemia the thymic tumor is small, or the thymus may not be enlarged at all. On the other hand, the spleen is usually considerably enlarged. The examination of the peripheral blood and of sections of internal organs, reveals the presence of white blood cells of the myeloid series, in different degrees of maturation.

The Passage A Mouse Leukemia Virus

It was determined over 10 years ago that leukemia developing spontaneously in certain inbred lines of mice is caused by a filterable and transmissible virus (9) From spontaneous Ak mouse leukemia a virus was isolated, whose pathogenic potency increased and became standardized after passage through newborn and suckling mice (11) Following inoculation of this virus, designated "passage A into suckling mice of a variety of susceptible strains (18) as well as into rats (19) the same spectrum of leukemia and lymphomas could be induced which has been observed to develop spontaneously in mice (16) The most frequently induced form was lymphatic. However under certain experimental conditions, in mice of certain strains (18) and particularly following thymectomy (14) myeloid leukemia could be induced as well as other less frequently occurring forms, such as chloroleukemia As tissue reactions, lymphosarcomas developed in most of the inoculated mice and rats reticulum-cell sarcomas developed less frequently but were also observed (18 19) It appears that the form of leukemia or lymphoma developing in the inoculated mice, or rats following inoculation of the passage A virus depends to a considerable extent on the genetic susceptibility of the host and on the activity of the thymus (14) the influence of the spleen seems to be of lesser importance (17)

It is now quite apparent that not only lymphoid leukemia and lymphosarcomas, but actually a wide spectrum of different forms of leukemia and lymphomas, usually occurring spontaneously in mice, could be induced in susceptible mice following inoculation of a single virus originally isolated from spontaneous mouse leukemia (table IV) This virus, which we have carried in cell free passage through newborn mice, and which we designated "passage A" is probably the prototype of the oncogenic virus responsible for the development of all the usual forms of leukemia and lymphomas in mice (fig 1)

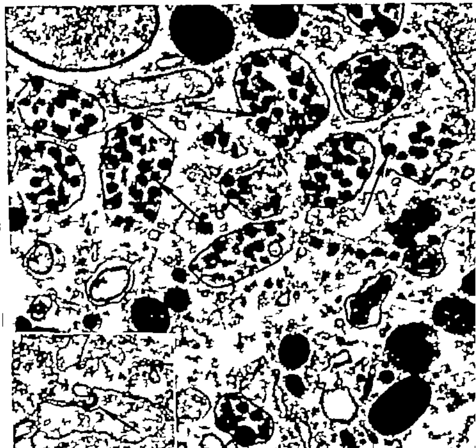


Fig. 2. Electron Micrograph of Passage A Mouse Leukemia Virus. Ultra-thin section of fragment of cervical lymph node from C3H(f) female mouse that developed leukem as result of inoculation of the passage A virus. Part of the cell is shown containing the cytoplasm several vacuoles filled with virus particles (arrows) × 33,885.
I the insert, there is part of cell from spleen of C3H(f) mouse with passage A virı induced leukemia, illustrating budding of particle (arrow) from the cell membrar × 38,000.

(Electron micrograph prepared in our laboratory by Dr. D. G. FELDMAN in cooperati with the author.)

Properties of the Virus

The properties, host range, and pathogenic potency of th

diameter which readily passes through bacterial filters. The virus does not resist heating to 50 C for 1 hour and can be inactivated in vitro by ethyl-ether (10) The mouse leukemia virus is pathogenic for a variety of strains of mice and also for rats. The virus can be also grown in tissue culture on normal mouse embryo cells (16) under such conditions, however it propagates only moderately either the mouse or the rat, is still preferable as a live medium for the propagation and harvesting of the virus.

Electron Microscopic Studies

The virus particles can be found in the cytoplasm of leukemic cells, in clusters filling out vacuoles (fig 2) or scattered in disintegrated cytoplasm frequently also considerable numbers of particles can be found in intercellular spaces. The particles can also be found leaving the cell's cytoplasm by budding and forming a villiform elongation of the cell membrane (fig 2 insert)

It is of interest that on electron microscopic examination of ultra-thin sections of leukemic organs of mice, or rats, virus particles could be found not only in leukemic cells (either forming lymphoid tumors, or infiltrating various organs such as spleens or livers) but also in apparently normal cells. Thus, considerable numbers of virus particles could be found in megakaryocytes and in blood platelets, in bone marrow and in spleen (4). More recently virus particles could be also found in normal free from leukemic cell infiltration, mammary glands and in milk ducts (5)

The virus particles are spherical and have an average diameter of approximately 100 mμ they have usually two membranes very few have three membranes. Those with double membranes, but without nucleoids, have the appearance of doughnuts, and are assumed to be immature virus particles. Particles with electron dense center i. e nucleoids considered to be mature virus particles, have usually one or two outer membranes, and can be found in vacuoles in cell cytoplasm, and also in intercellular spaces.

Leukemic virus particles have been found in at least some of the organs of *all* leukemic mice and rats with passage A virus-induced leukemia thus far examined Considerable numbers of virus particles have been found in thymus, bone marrow spleen and lymph nodes of these animals. Among other organs, virus particles were found in kidneys, livers and lungs of leukemic animals, but only in leukemic cells infiltrating such organs.

Electron microscopic examination of normal, healthy mice of nonleukemic strains, such as C3H, revealed the presence of only isolated single particles in some of the organs examined, particularly in the thymus. With only very few isolated exceptions, none of

the particles found in the normal control animals had an electron-dense central nucleoid; particles found in healthy mice of low-leukemic strains were of the immature doughnut type. It should be emphasized that no particles hav thus far been found in normal organs of healthy non-injected rats.

The consistent presence of large numbers of characteristic particles in organs of all mice and rats with virus-induced leukemia is suggestive, and contrasts sharply with only ery occasional presence of isolated particles in certain organs (particularly thymus) of normal mice, and apparent total absence of similar particles in tissues of normal rats thus far examined. At the present time, nevertheless, there is only circumstantial evidence to suggest that the submicroscopic particles here described actually represent the mouse leukemia virus.

The Mouse Leukemia Virus Strain Isolated by Graffi

In extensive studies, Graffi (6 7 8) and his co-workers made the important observation that cell-free extracts prepared from a variety of transplanted mouse tumours could induce leukemia following inoculation into newborn mice of susceptible strains. Some of theses tumors yielded a considerable quantity of a potent leukemic virus.

In September 1963 we received from Dr A. Graffi four mice with leukemia induced with his virus strain. From leukemic lymph nodes, fragments of thymic tumors, spleens, and livers of these mice, cell suspensions were prepared, and centrifuged in the usual manner

Table I

Incidence of leukemia induced in C3H(f) and BALB/ mice following inoculation of the graffi irus strain.

Strain	No. of mice inoc.	No. dev. leuk.	Leuk. incid. %	Aver. age leuk. dev. mon.
C3H(f)	29	25	86	2.9
BALB/c	21	20	95	2

C3H(f) mice of Bittner subline of strain C3H, free from mouse mammary carcinoma virus by foster nursing.

All mice inoculated i. p. (0.2 to 0.3 ml each) when 2 to 6 days old.

(11) and the supernate was then passed through Selas, 02 filter candles. The filtrate of 10 / concentration thus obtained was inoculated intraperitoneally (0.2 to 0.3 ml each) into suckling less than 6 days old, mice of strains C3H(f) and BALB/c. As soon as some of the inoculated mice developed leukemia, they were used as donors for the preparation of additional filtrates, which were again inoculated into suckling mice of both strains. Most of the inoculated mice developed leukemia after 2 to 3 months (table I)



Fig 3. Mouse Leukemia Induced ith the Graffi Strain of the Mouse Leukemia Virus. This BALB/c female was inoculated (0.2 to 0.3 ml i. p.) when less than 4 days old with the Graffi mouse leukemia virus strain filtrate ($10^{-?}$) and developed generalized leukemia at the age of 2? months. Note the very large thymic tumor ery large spleen and liver and also large peripheral lymph nodes. On microscopic examination of blood smears the form of leukemia was diagnosed as myeloid.

A preliminary titration experiments, carried out on 2 to 6 days old C3H(f) mice $10^{-?}$ and $10^{-?}$ dilutions of the filtrate induced 100% and 91 incidence of leukemia, respectively the incidence dropped to 50% when 10^{-4} dilution was inoculated. Only one out of 7 mice developed leukemia, following inoculation of $10^{-?}$ dilution of the filtrate

Leukemia induced in our laboratory with the Graffi virus strain in mice of strains C3H(f) and BALB/c was for all practical purposes indistinguishable from that resulting from inoculation of the passage A virus. Most of the leukemic mice had considerably enlarged spleens and livers, large mesenteric tumors, and frequently also enlarged peripheral lymph nodes (fig 3) thymic tumors were present in practically all leukemic mice of the C3H(f) strain, but only in some of the BALB/c mice. Microscopic examination of blood and tissues of the leukemic mice revealed either lymphatic, stem cell or myelogenous leukemia (table II) this latter form was quite

Table II

Forms of leukemia developing in C3H(f) and BALB/c mice following inoculation of graffi virus strain.

	No. of leuk. mice examined	No. dev. lymphat. leuk.	Lymphat. leuk. incid. %	No. dev myeloid leuk.	Myeloid leuk. incid. %	No. dev stem-cell leuk.	Stem-cell leuk. incid.
C3H(f)	28	21	75	0	0	7	25
BALB/	19	11[1]	58	5	27	3	15

18 of 21 mice (64%) in the C3H(f) group, and 8 of 11 (42%) in the BALB/c group, had the aleukemic form of lymphatic leukemia, i. e. generalized lymphosarcoma, with no significant changes in peripheral blood morphology

frequently observed in BALB/c mice, as was the case also in our previous studies in which leukemia had been induced with the passage A virus (18) however in our mice inoculated with the Graffi virus strain, we have not seen chloroleukemia, a form quite frequently observed by Graffi in his laboratory

Graffi reported that this virus strain was sensitive to treatment in vitro with ethyl-ether (7) In this respect the Graffi virus strain was not different from the passage A virus (10)

The sensitivity of the Graffi virus strain to moderate heating was also the same as that of the passage A virus (15) After heating the Graffi virus strain filtrate, in water bath to 50 °C for ½ hour we inoculated (p. 0.3 ml each) 6 suckling C3H(f) mice with the heated filtrate, and all remained in good health. Six control mice of the same strain and age, inoculated simultaneously with fresh filtrate developed leukemia when they reached 2½ to 3 months of age.

Preliminary electron microscopic studies of ultra thin sections of organs of mice that developed leukemia as a result of inoculation of the Graffi virus strain revealed the presence of spherical particles not different from the passage A virus (fig 4) their size, morphology and location were the same as those observed in our previous studies dealing with passage A virus-induced leukemia.

The Mouse Leukemia Virus Strain Isolated by Moloney

Following the studies of Graffi, a potent leukemogenic virus strain was isolated by J B Moloney from Sarcoma S 37 a tumor that had been transplanted for many successive generations in mice (27 28)

In October 1962, we received from Dr Moloney several BALB/c mice inoculated with his virus strain. As soon as these mice developed leukemia, we prepared filtrates from their leukemic

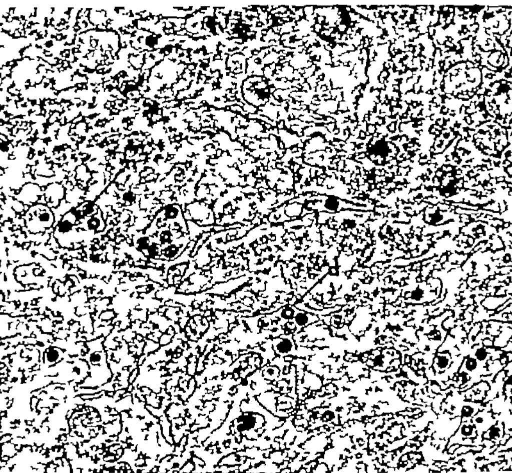


Fig 4 Electron Micrograph of Gross Strain of the Mouse Leukemia Virus. Ultra-thin section of megakaryocyte from spleen of BALB female mouse that developed leukemia as result of inoculation with the GRAFFI virus strain filtrate. Within the vacuoles of the cell cytoplasm are groups of particles, some of them of the doughnut-type (d), and others containing nucleoids ( ) 33,880.
Electron micrograph prepared in our laboratory by Dr D. G FELDMAN in cooperation with the author)

organs, using the method routinely employed in our laboratory for the preparation of passage A virus extracts, and previously described 11) the same which we also employed for the preparation of the GRAFFI virus strain filtrates (see page 49)

This simple method of preparation of a filtrate was considerably less time consuming, than the differential centrifugation method employed by MOLONEY (27)

KIRSTEN 3 compared recently both methods of preparation of extracts, using spontaneous Ak leukemias as source material, and newborn rats as test animals, and

concluded that the difference between the method employed in our laboratory and the differential centrifugation method employed by MOLONEY was not significant, since the incidence of leukemia induced in rats with extracts prepared by the differential centrifugation method was 25 as compared with the 18 to 20* incidence obtained by the method employed in our laboratory

The filtrate was inoculated into suckling 2 to 6 days old, mice of strains C3H(f) and BALB/c. Of 74 mice of strain C3H(f) 72 (97 /) developed leukemia at an average age of 4 2 months. Twenty four BALB/c mice were inoculated, and all of them (100 /) developed leukemia at an average age of 3 4 months. In preliminary titration experiments, 10^{-2} and 10^{-3} dilutions of the filtrate inoculated into 2 to 6 days old mice of either strain induced a 100 / incidence of leukemia additional experiments with higher dilutions are now progress.

Leukemia induced in C3H(f) or BALB/c mice with the MOLONEY strain (fig 1) was indistinguishable from that induced in mice of either inbred line with the passage A virus (fig 1) Most of the leukemic mice had large thymic tumors, considerably enlarged spleens and livers, large mesenteric tumors and frequently also large peripheral lymph nodes. The spleens were particularly large in mice of strain BALB/c with virus-induced leukemia. Microscopic examination of blood smears and tissues of 21 C3H(f) leukemic mice revealed that 14 had the lymphatic form (12 among these had generalized lymphosarcomas with no evidence of leukemia in peripheral blood morphology) and 7 had stem-cell leukemia. Among 14 leukemic BALB/c mice examined 6 had lymphatic leukemia (5 of these had generalized lymphosarcomas) 2 had stem-cell leukemia and 6 (43 /) had myeloid leukemia. Briefly the forms of leukemia induced with the MOLONEY virus strain did not essentially differ from those induced with the passage A virus.

Certain physical properties of the virus strain isolated by MOLONEY have been studied in our laboratory again they were found indistinguishable from those found in our previous studies carried out with the passage A virus. In our experiments the MOLONEY virus strain was inactivated by heating to 50 C for 30 minutes the same was true for the passage A virus (15) We also found that the MOLONEY virus strain is sensitive to in vitro exposure to ethyl ether (table III)

This observation was in contrast to previous brief statement made to the effect by MOLONEY (28) However it is quite possible that in single experiment, some of the

L. GROSS Unpublished data (1963).

Table III

Influence of ethyl-ether on the leukemogenic potency of the moloney strain of the mouse leukemia virus.

Ether-treated Filtrate[1]					*Controls: Filtrate only*[2]				
Inbred line of mice	No. of mice inoc.	No. dev. leuk.	Leuk. incid. %	Average leuk. dev. mos.	Inbred line of mice	No. of mice inoc.	No. dev. leuk.	Leuk. incid. %	Average leuk. dev. mos.
BALB	22	—	—	—	BALB/c	18	17	95	< 4
C3H(f)	50	6	12	6	C3H(f)	41	38	93	4
	72	6	8	6		59	55	93	4

I 6 separate experiments, 5 to 10% leukemic filtrate was mixed, and vigorously shaken, with ethyl-ether (adding 2 ml of ether to each tube containing either 5 6, or 8 ml of filtrate) The mixture was left overnight t 0 C in refrigerator The ether was then evaporated in flask, using gentle suction under a vacuum pressure of approximately 20 mm of mercury

The controls consisted of the same filtrate, 5, 6, or 8 ml in each tube, mixed with 2 ml of physiological saline solution, instead of ether The control mixtures were also left at 0 C overnight and then submitted to an evaporation procedure similar to that employed for the ether-containing samples.

Suckling mice 2 to 6 days old, inoculated i. p. 0.2 to 0.3 ml each.

Mice which survived, and did not develop leukemia, were observed until they reached about 8 months of age

virus could have survived treatment with ether inducing subsequently leukemia in some of the inoculated animals in more extensive study such as that carried out in our laboratory most of the Moloney virus strain was destroyed by ether as was the case in similar experiment carried out previously with the passage A virus (10)

Electron microscopic studies of ultra thin sections of organs of mice that developed leukemia as a result of inoculation of the Moloney virus strain, revealed the presence of spherical particles not different from the passage A virus their size, morphology and location (fig 5) were the same as those observed in our previous studies dealing with passage A virus-induced leukemia (4 5 16)

The Virus Strain Isolated from Radiation-Induced Leukemia

A filterable leukemogenic agent isolated from radiation-induced mouse leukemia (12) and designated "passage X" has been passed through suckling mice of either C3H(f) or C57 Brown/cd strains, inducing leukemia in most of the inoculated animals after an average latency varying from 5 to 8.5 months (13) In its more recent 13th to 16th consecutive cell free passage through suckling C3H(f) mice this virus strain induced leukemia mostly of the lymphatic form, in 54 out of 62 inoculated mice (87 /) at an average

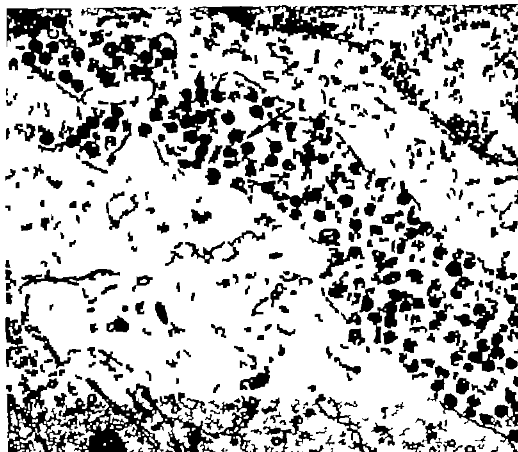


Fig. 5. Electron Micrograph of Moloney Strain of the Mouse Leukemia Virus. Ultra-thin section of fragment of spleen from C3H(f) female mouse that developed leukemia as result of inoculation with the Moloney virus strain filtrate. Considerable number of typical spherical virus particles in intercellular spaces (arrows) × 34,000.
(Electron micrograph prepared in our laboratory by Dr G. D. Feldman in cooperation with the author.)

age of 7 months. Leukemia induced in mice with the passage X virus strain was indistinguishable from that induced under similar experimental conditions with the passage A virus. Preliminary electron microscopic studies of ultra-thin sections of organs of mice that developed leukemia as a result of inoculation of the passage X virus strain, revealed the presence of spherical particles (fig. 6) identical in size, morphology and location with those of the passage A virus (4, 5, 16).

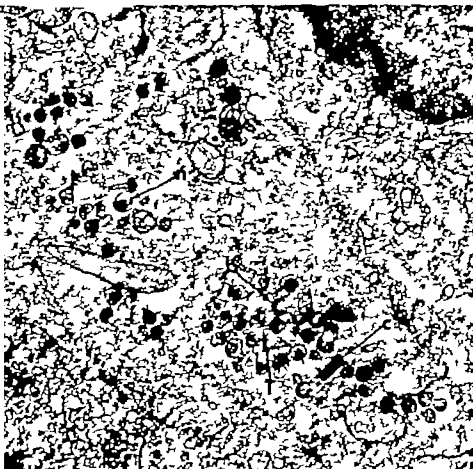


Fig 6. Electron Micrograph of Passage X (Radiation-Induced) Mouse Leukemia Virus. Ultra-thin section of a fragment of peripheral lymph node from C3H(f) mouse that developed leukemia as result of inoculation of the passage X virus. The intercellular space contains several types of particles, budding (b), cylindrical ( ) with nucleoids (n) and with "tails (t) 33,880.
(Electron micrograph prepared in our laboratory by Dr D. G. FELDMAN in cooperation with the author)

A filterable leukemogenic virus was also isolated in mice from radiation induced leukemia by LIEBERMAN AND KAPLAN (26) and also by LATARJET AND DUPLAN (25) More recently JENKINS AND UPTON (21) induced myeloid leukemia by radiation in mice of the RF strain from such radiation-induced leukemia a virus could be isolated which, following inoculation into newborn mice of the RF strain, induced leukemia predominantly of the myeloid form.

Whether the ability of this virus strain to induce myeloid leukemia is a characteristic feature of this particular virus, or whether it is related to the inbred line of mice employed for inoculation, remains to be determined.

How Many Viruses Causing Leukemia and Lymphomas in Mice?

After the experimental conditions were determined under which a leukemic virus could be isolated in mice, it became gradually a routine procedure to follow the established technique, and to isolate leukemogenic viruses from spontaneous mouse leukemia more recently it also became apparent that similar leukemogenic viruses could be isolated without difficulty from other sources, particularly from transplanted mouse tumors (6, 8) A considerable number of individual isolations of leukemogenic viruses in mice has been reported from many laboratories (1 2 3 6 8, 12 21 22 25 26, 27 28, 30 31 32 33 35)

The question arises whether these leukemogenic agents, isolated from different sources, represent individual and distinct viruses, or whether they represent isolations from different sources of the same leukemic virus, or at best one of its close variants.

Under otherwise similar experimental conditions, i. e. using mice of the same inbred line as test animals, the disease induced with either of the several available virus strains, such as the passage A virus, the passage X, Graffi and the Moloney strains, etc., is indistinguishable on either macroscopic or microscopic examination the morphology is basically the same. We are, therefore, faced with the fact that a specific complex of symptoms, reflecting the usual forms of mouse leukemia and lymphomas, can be induced in mice and rats with a number of filterable virus strains isolated from a variety of sources in the mouse, i. e. from spontaneous mouse leukemias, from radiation induced leukemia, and from transplanted mouse tumors.

It should be also stressed that the mouse leukemia virus, even though isolated from different sources, has identical physical properties. The virus is a spherical particle about 100 mμ in diameter and can be found in the cytoplasm or in the intercellular spaces, of virus-infected cells. The leukemic filtrates containing the virus particles are thermolabile, and ether-sensitive.

Table II

Forms of leukemia and lymphomas induced in mice and rats with mouse leukemia virus type A.

Leukemia

Lymphatic Leukemia
- a) aleukemic
- b) leukemic

Stem-cell Leukemia

Myelogenous Leukemia
- a) undifferentiated
- b) well differentiated

Chloro-Leukemia

Erythroblastic leukemia (atypical)

Monocytic-like leukemia

Lymphomas

Lymphosarcomas
- a) local lesion
- b) generalized

Reticulum-cell sarcoma

Hodgkin s like lesions

It is quite apparent that our present means serving for identification of closely related oncogenic virus strains are limited This difficulty is increased by the fact that under experimental conditions thus far employed certain oncogenic viruses, and particularly the mouse leukemia virus, have demonstrated a relative lack of antigenic potency

There is some possibility now investigated, to suggest that under certain experimental conditions slight immunological differences can be found among at least some of the leukemogenic virus strains, such as between the passage A virus, and the MOLONEY virus strain (24 29) However it should not be surprising to find minor immunological differences between such virus variants in many other virus diseases, individual virus strains isolated from different sources have been found to be immunologically distinct. Such variants represent, nevertheless, the same virus. Among oncogenic viruses, one could mention, as an example, the several variants of the Rous sarcoma virus (34) or the immunologically distinct variants of the polyoma virus recently described (20) such

variants may be distinct antigenically some of them may even show differences in their pathogenic potency evidenced on inoculation tests, or in tissue culture. Viruses are prone to mutate and to form variants such variants do not necessarily need to be permanent.

The Mouse Leukemia Virus Type A

On the basis of experimental data thus far available it appears reasonable to assume that most of the individual isolations of leukemogenic viruses in mice, represent recoveries, from different sources, of the same leukemia virus, or at best one of its closely related variant strains either of them may induce in mice the same disease. Even though some of such virus strains may show minor immunological differences, it is only reasonable to assume that they all represent basically the same virus. It seems that it would be useful to designate this basic mouse leukemia virus by a symbol for that reason it is suggested that the mouse leukemia virus, and its related variants, which induce in mice the typical and well known picture of mouse leukemia, be designated as the "Mouse Leukemia Virus Type A"

It should be stressed that the lymphatic form of leukemia, although most frequent, is not the only one that can be induced with the mouse leukemia virus type A. The same virus may induce not only lymphatic leukemia, but also acute stem-cell leukemia, myelogenous leukemia, and in certain instances also the more rare forms, such as chloroleukemia, etc. and as tissue reactions not only lymphosarcomas, but also reticulum-cell sarcomas, and Hodgkin's-like lesions (table IV)

If this assumption is correct the great majority of the usual forms of mouse leukemia and lymphomas, observed to develop "spontaneously" (9) or induced by radiation (12) or carcinogenic chemicals (36) in mice of different strains, would be caused by the same virus, i. e. the mouse leukemia virus type A, or by one of its close variants*

A different form of disease of the hematopoietic system, which also belongs to the broad group of leukemias, is caused by virus isolated by C. FRIEND; the leukemia virus strain isolated more recently by RAUSCHER appears to be very similar to, if not identical with, the FRIEND virus. The disease induced by either the FRIEND virus or the RAUSCHER virus strain, and its possible relation to the Mouse Leukemia Virus Type A, will be discussed by the author in separate paper in the next issue of Acta haemat.

Table IV

Forms of leukemia and lymphomas induced in mice and rats with mouse leukemia virus type A.

Leukemia

Lymphatic Leukemia
- a) aleukemic
- b) leukemic

Stem-cell Leukemia

Myelogenous Leukemia
- a) undifferentiated
- b) well differentiated

Chloro-Leukemia

Erythroblastic leukemia (atypical)

Monocytic-like leukemia

Lymphomas

Lymphosarcomas
- a) local lesion
- b) generalized

Reticulum-cell sarcoma

Hodgkin s-like lesions

It is quite apparent that our present means serving for identification of closely related oncogenic virus strains are limited. This difficulty is increased by the fact that under experimental conditions thus far employed certain oncogenic viruses, and particularly the mouse leukemia virus, have demonstrated a relative lack of antigenic potency.

There is some possibility, now investigated, to suggest that under certain experimental conditions slight immunological differences can be found among at least some of the leukemogenic virus strains, such as between the passage A virus, and the Moloney virus strain (24, 29). However, it should not be surprising to find minor immunological differences between such virus variants. In many other virus diseases, individual virus strains isolated from different sources have been found to be immunologically distinct. Such variants represent, nevertheless, the same virus. Among oncogenic viruses, one could mention, as an example, the several variants of the Rous sarcoma virus (34) or the immunologically distinct variants of the polyoma virus recently described (70), such

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Société Européenne d Haematologie

Le *Ve Congrès de la Société Européenne d'Hématologie* se tiendra à Strasbourg (France) du 23 au 28 août 1965.

Les *principaux sujets* traités seront La structure des protéines sanguines et des hémoglobines. L étiologie et la chimiothérapie des leucémies. Le premier temps de l'hémostase. L'hémophilie et sa thérapeutique. Les problèmes génétiques en hématologie L'hématologie géographique. La chimie de substances de groupe. Les enzymes érythrocytaires. Aspects morphologiques et fonctionnels des lymphocytes.

Certaines séances seront consacrées à des problèmes intéressant plus particulièrement la transfusion (séparation des éléments figurés du sang conservation du sang et de la moelle osseuse à basse température, etc.)

Tous renseignements complémentaires seront fournis par le Dr J Lewis, Centre de Transfusion Sanguine, 10 rue Spielmann, Strasbourg (France)

Libri

M. A. Atamer Blood Diseases. Grune & Stratton Inc., New York 1963 618 p., Price $ 16.50.

This book reflects the growing trend to use the computer for aiding differential diagnosis, prognosis and therapy of diseases. The author has analyzed and recorded the information on 18,000 cases of 75 hematologic disorders, reported in the literature One can not help feeling it highly questionable whether such book recommendable and appropriate way of presenting hematology—as the uthor intends— in concise form for the medical student and practising physician The text concerning the various diseases is short; main features are the tables at the end of each chapter which list (from selected references) symptoms, clinical findings and the mean percentage frequently based on few cases. Atypical cases with unusual manifestations (often important and leading signs!) are in the words of the author carefully eliminated' Neither students nor physicians will successfully learn clinical and every days hematology from this book; it is even doubtful, whether it is desirable addition to the existing shorter or more comprehensive good text books.

The unfortunate tendency of prospective uthors to use reviews and summaries instead of reading the earlier original publications on the respective topic may be enhanced by presentation of this type G. Rosenow New York

P. Croizat, L. Revol et P. Morel, Savoir Interpréter No. 12. L'Examen Hématologique. Éd. par Albert de Vaucher. Maloine, Paris 1964.

Der obige Verlag gibt unter dem Motto „Savoir Interpréter" eine Reihe von Bändchen heraus, die in übersichtlicher und knapp gefaßter Form dem praktisch tätigen Arzt die wichtigsten Befunde und heutigen Erkenntnisse der verschiedenen Organerkrankungen „ermitteln". Aus dieser bei den französisch sprechenden Ärzten sehr beliebten Buchreihe ist nun von den obigen Autoren, die eine führende Rolle unter den französischen Hämatologen bilden, ein Bändchen über „L'Examen Hématologique" herausgekommen. Der allzu früh verstorbene Professor Lambert, Louvain, hebt in seinem prägnanten Vorwort hervor, daß das vorliegende Büchlein auf Grund der großen klinischen Erfahrung dieser Autoren an der medizinischen Universitätsklinik Lyon an einem großen Patientenmaterial aufgebaut wurde. Diese klinische Erfahrung spürt man in der Niederschrift der verschiedenen Kapitel immer wieder heraus, und so kann das kleine, prägnant gefaßte und auf den modernsten Stand gebrachte Büchlein dem praktischen Arzt als rasches Nachschlage- und Orientierungswerk auf das allerbeste empfohlen werden. Es ist nicht zu zweifeln, daß es zahlreiche Leser und zahlreiche Auflagen erleben wird.

S. Moeschlin, Solothurn

Ch. G. de Boroviczény: Erythrocytometric Methods and their Standardization. Transactions of the Standardizing Committee and Proceedings of Symposium XVIII of the Ninth Congress of the European Society of Haematology, Lisbon 1963. Bibliotheca Haematologica, Fasc. 18. S. Karger, Basel/New York 1964. VI + 124 S., 24 Abb., 23 Tab., Preis sF/DM 19.

Der Herausgeber bemüht sich seit einigen Jahren in verdankenswerter Weise um eine Vereinheitlichung hämatologischer Untersuchungsmethoden. Diesem Ziel diente auch das am Hämatologen-Kongreß in Lissabon veranstaltete Symposion, dessen Vorträge und Diskussionsvoten hier veröffentlicht werden. Es handelt sich um 16 Referate über elektronische Erythrocytenzählung, Hämatokritbestimmung, Messungen des Zelldurchmessers, Bestimmung der Hämoglobinkonzentration und über bisherige Standardisierungsbestrebungen. Kompetente Fachleute besprechen methodische Probleme, mit denen sich jeder Leiter eines hämatologischen Laboratoriums auseinandersetzen muß. Am Schluß des Treffens wurde ein Standardisierungskomitee der Europäischen Gesellschaft für Hämatologie ins Leben gerufen, das alle nationalen europäischen Hämatologen-Gesellschaften auffordert, sich für die Durchführung der Standardisierungsbeschlüsse einzusetzen. Präzise Empfehlungen liegen bereits für die Hämoglobinbestimmung vor: Die Hämoglobinkonzentration soll in Milliäquivalent pro Liter (mval) oder in Gramm pro 100 ml (g%) angegeben werden. Die Bestimmungsmethode der Wahl ist die spektrophotometrische Messung von Hämoglobincyanid bei 540 mμ, wobei der Berechnung ein millimolarer Extinktionskoeffizient von 11,0 zugrundegelegt werden soll. Zur Messung wird eine 251fache Blutverdünnung empfohlen. Hämoglobincyanid-Standardlösungen sind in Ampullen jahrelang haltbar und können u. a. vom Standardisierungskomitee bezogen werden.

Der preiswerte kleine Band enthält viele nützliche Angaben und methodische Hinweise und kann jedermann empfohlen werden, der sich mit hämatologischen Laboratoriumsuntersuchungen beschäftigt.

H. R. Marti, Basel

Acta haemat. 32. 65–80 (1964).

From the Department of Medicine and the Radioisotope Laboratory of the Burgerspital, Solothurn (Chief Prof. S. Moeschlin)

Pernicious Anaemia

An Erythrokinetic and Autoradiographic Study Using H^3-Thymidine, H^3 Uridine and H^3-Cytidine*

By Jakob R. Schmid Sven Moeschlin and Viktor Hagi**

In spite of the fact that the malignant course of pernicious anaemia (P A.) has become abruptly reverted by Minot's (27) introduction of liver therapy in 1926–1929 and Castle's (8–11) basic studies in 1929—1931 and in spite of the recognition of vitamin B_{12} in 1948 as Castle's postulated extrinsic factor (5) that requires a gastric mucoprotein (intrinsic factor) for its reabsorption, many problems remain to be solved. While biologic functions of vitamin B_{12} and folic acid certainly are closely interrelated their metabolic basis is still almost entirely unknown. Further progress has been made predominantly in the therapeutic field of the disease by the introduction of a microbiologic assay using Lactobacillus casei. Its application permitted Rickes et al. (38) to isolate in 1948 the cristalline substance of B_{12} from the liver Subsequently the parenteral route became the application of choice (53) and synthetic products were available after knowledge of the vitamin's structure in 1955 (6, 17)

In haematology progress was made from Naegeli's (29) and Ferrata's (12) historical concept of two independent normoblastic and megaloblastic lines originating from separate stem cells. Today an interconversion of normoblastic and megaloblastic forms is generally assumed to occur on the basis of some biochemical alteration, as yet to be exactly defined. While the pathogenetic mechanism leading to megaloblastosis has remained poorly understood,

* This study was supported by grant of the Swiss National Foundation for Scientific Research (No. 2696).

** With technical assistance of Mrs. U Gerber

studies of the bone marrow in tissue culture, especially with the use of isotopes and autoradiography opened a new field to investigate the problem. Certain aspects of nucleic acid metabolism, e. g. DNA and RNA synthesis of cells may thus be correlated with morphologic changes. The present article is intended to demonstrate such possibilities by the detailed analysis of an individual case.

Patients

Case of pernicious anaemia. S. J. (file no. 9067/834) a 62 year-old labourer was referred to the Clinic on July 22, 1963 for evaluation of anaemia of recent onset. The past history was non-contributory. Physical examination was unremarkable except for marked waxy pallor and some evidence of glossitis. The neurological status was unrevealing. On admission, the haemoglobin was 6.7 g %, erythrocytes 1.28 million/cu. mm, leucocytes 600 cu. mm with normal differential count, platelets 230'000/cu.mm, serum bilirubin 1.55 mg per 100 ml, serum iron 126 μg per 100 ml and reticulocytes 1.7 %. On a peripheral blood smear red cells were megalocytic with aniso-poikilocytosis and occasional stippling and neutrophils were right-shifted. Examination of bone marrow smears revealed greatly increased and purely megaloblastic erythropoiesis in which basophilic forms predominated. Granulocytopoiesis was left-shifted with giant metamyelocytic cells being plentiful, in addition. Except for histamine-fast achlorhydria, the remaining laboratory studies were found to be within normal limits.

Following parenteral vitamin B_{12}-therapy a reticulocyte response reaching a peak of 23.7% was observed on the 7th day. Subsequently the haemoglobin level rose to 11.6 g% prior to the patient's dismissal.

Control subjects. Cr^{51}-labeled erythrocytes of the patient with P.A. were transfused to control subjects. These normal subjects were examined regarding blood group, subgroups and cross matches in the indirect Coombs serum and found to be homologous with the patient S. J.

Materials and Methods

Erythrokinetic methods. Erythrocyte survival and red cell volume were measured with radiochromium using the method described by Sterling and Gray (44), in the modification of Read (35). Plasma iron clearance and plasma and red cell iron turnover were determined according to the procedure of Huff et al. (18). The Fe^{59} and Cr^{51} radioactivity in the daily blood samples were determined by spectrometric analyses in one-channel well type scintillation counter with two-inch sodium-iodide (Tl) crystal. The latent serum iron-binding capacity was measured according to the method of Tauxe (45) using radioiron.

Autoradiographic method. Exactly known volumes of marrow samples were pipetted immediately following sternal aspiration into siliconized incubation containers. By means of lambda pipettes, H^3-thymidine, H^3-uridine and H^3-cytidine (spec. act. 1.9 C/mM) were added and the samples were incubated at 37° C with constant gentle shaking for a 1-hour period. This procedure was used to keep the concentration of the radioactive reagent constant at 0.8 μc/ml in each marrow-isotope sample. At the end of one hour smears were prepared from each sample on subbed slides, air dried and fixed in methanol. They were further processed in the dark, using Kodak AR 10 stripping film according to the technique described by Pelc (30). The mounted slides were air dried, alcohol dried and subsequently stored in a refrigerator. Slides made from marrow samples incubated with H^3-thymidine were developped after a period of exposure of 5–8 days, those from samples incubated with H^3-uridine or H^3-cytidine at regular

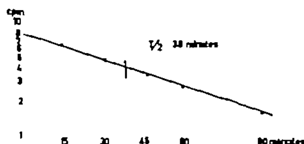


Fig 1 Plasma clearance of Fe^{59}



Fig 2 Fe^{59} incorporation into erythrocytes over 14 days, rate of incorp. approx. 47%

5-day-intervals over total period of at least 30 days. The stripping film was developed at 18° C using Kodak D-19 developer and Kodak acid fixer For staining with Giemsa, the slides were placed in solution of 18 °C, buffered at pH 6 5.

Slide evaluation was based on counting the per cent labeling for each cell type. As parameter for the incorporation of H^3-uridine and H^3-cytidine, percentage counts were considered to give basically the same information as grain counts. A cell was defined as labeled if the number of grains over the evaluated area (nucleus) was greater than twice background.

Morphologically the criteria stated by WITTMOSS (53) were used to classify megaloblasts. However no attempt was made to evaluate in the autoradiograms promegaloblasts separately from basophilic megaloblasts, and no distinction was made between early and late polychromatophilic forms.

Results

Erythrokinetics Data of the Cr^{51} and Fe^{59} studies in the patient with pernicious anaemia (P A.) are summarized in table I Reduction of the red cell mass to 17.35 ml/kg was an expression of severe anaemia. Half clearance time of plasma iron (fig 1) was accelerated with 38 0 (our normal limits 60–100) minutes, and plasma iron turnover rate increased to 102 15 (normal 30–40) mg/70 kg/day The rate of incorporation of Fe^{59} into newly formed

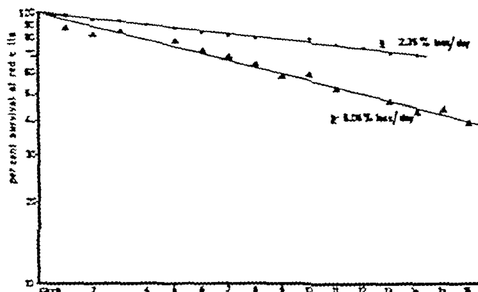


Fig. 1. Cr^{51} survival of red cells in the patient with P.A. a: following, and b: prior to B_{12}-therapy. Treatment corrected the increased rate of erythrocyte hemolysis (5.06 % loss/day) to normal value (2.35 % loss/day).

Table I
Erythrokinetic data.

	Patient	Normal Range
Serum iron, µg/100 ml	126	80–150
Latent iron binding capacity µg/100 ml	134.5	150–250
Haematocrit (corrected),	23.0	
Blood volume (Cr^{51}) ml/kg	63.4	70±10
Red cell mass (Cr^{51}), ml/kg	17.55	30±4
Plasma volume (Fe^{59}) ml/kg	37.7	40±5
Plasma iron clearance T/2, minutes	38.0	60–120
Plasma iron turnover rate, mg/day	102.1	30–40
Fe^{59} incorporation into red cells,	4 7	80–95
Red cell iron turnover rate, mg/day	48.7	23–35

Red cell survival studies (Cr^{51}) Per cent — Normal values: less than 2.5%

with P.A. prior to B_{12}-therapy — 5.06%/day
with P.A. following B_{12}-therapy — 2.35%/day
of untreated patient with P.A. — homologous subject — /day
patient with P.A. — subject sub- B_{12}-therapy — /day

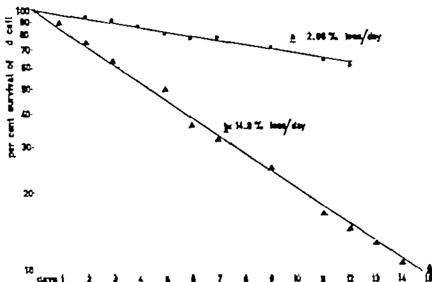


Fig 4 Cr^{51} survival of P.A. erythrocytes transfused to an homologous normal subject; *a.* red cells of the treated patient are destroyed at the practically normal rate of 2.86%/day while *b* red cells transfused prior to B_{12}-therapy are rapidly destroyed in the control subjects (14.8%/day).

erythrocytes at the end of 14 days (fig 2) was low with only 47 (*normal more than 80*) % The amount of blood produced and destroyed daily calculated from the Fe^{59} and Cr^{51} data, indicated that the excessive loss had almost been compensated in this patient, although at low haemoglobin and red cell levels (table I)

Survival studies with radiochromium prior to B_{12} therapy (fig 3a) gave evidence of markedly increased erythrocyte haemolysis (5.06% loss per day) Following parenteral vitamin B_{12} therapy survival of red cells measured in the patient's own circulation were found to be within normal limits (fig 3b)

Red cells of the untreated patient with P A. injected to a homologous subject, were found to be destroyed randomly at the rapid rate of 14.8% per day (fig 4a) while subsequent to B_{12} therapy these cells were destroyed in a homologous subject at an almost normal rate with 2.86 % loss per day (fig 4b)

In vitro 1-hour DNA synthesis index Data about the short-term DNA synthesis index (H^3-thymidine) expressing the proliferative potential of cells, are summarized in table II. Highest values were noted in basophilic megaloblasts with an index of 74% and in

Table II

The one-hour DNA synthesis index (H^3-thymidine) of bone marrow cells in a patient with pernicious anaemia. (200 cells counted of each type).

Cell type	Labeled cells, %
Megaloblasts, basophilic	74
polychromatophilic	54
orthochromatic	0
Myelocytes	47
Metamyelocytes	21
Neutrophils	0
Small lymphocytes	0
Megakaryocytes (40 cells)	0

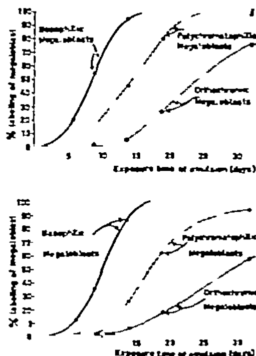


Fig. 5 and 6. Incorporation of H^3-uridine and H^3-cytidine into megaloblasts. The curves express the percentage increase in labeling as function of the exposure time of the photosensitive stripping film. Spec. Act. 1.9 C/mM, concentr. in the incubation medium 0.8 μc/ml.

polychromatophilic forms with 54% labeling. P.A. myelocytes were labeled in 47 and giant P.A. metamyelocytes in 21%. No labeling was found in orthochromatic megaloblasts, small lymphocytes, megakaryocytes and all the mature granulocytic cell types. The nuclear pattern of labeling with H^3-thymidine may be seen from fig. 7a.

In vitro 1-hour RNA synthesis rate Using H^3-uridine and H^3 cytidine, the per cent increase in labeling is expressed graphically for the different megaloblastic cells in fig 5 and 6 as a function of the exposure time of the photosensitive film. According to their steepest slope, basophilic megaloblasts incorporate both predominant RNA precursors at a higher rate than polychromatophilic cells. A small amount of incorporation of these precursors was also noted to occur in orthochromic forms. No significant difference in the intensity of incorporation was found by the use of H^3 uridine and H^3-cytidine. The pattern of labeling may best be recognized from the fig 7b–7d it is characterized for both precursors by its strict limitation to the nuclear area following a brief 1-hour incubation period, as applied in the present study

Discussion

Erythrokinetic aspects Our erythrokinetic studies demonstrated an increased rate of destruction of megalocytes. This finding was first postulated by HUNTER (19) in 1907 and has repeatedly been confirmed since by the use of different methods (20, 33 42, 46) The observations that megalocytes haemolyzed in the untreated patient and even more rapidly when injected to a normal control subject (fig 3a und 4a) while normal survival rate was measured following vitamin B_{12} therapy (fig 3b and 4b) are indicative for an intra corpuscular defect of these cells. Further evidence for this conclusion has been forwarded in the past from several experiments (20 33 46)

The haemolytic component in P A. has further been clarified in recent years by HAMILTON et al. (14) who demonstrated, in addition, an extracorpuscular factor vitamin B_{12} deficiency itself. Evidence for this was given from two observations. Normal red cells were found to have a decreased survival rate following injection to patients with P A. Furthermore, normal erythrocytes were destroyed in normal subjects more rapidly following a 6-hour in vitro contact with P A. serum than cells brought in contact with normal serum. Our own results confirm those of HAMILTON et al. (15 16) demonstrating that red cells of patients with P A. had normal survival rates when injected to normal subjects after B_{12} therapy (fig 4b)

The considerable increase in the plasma iron turnover rate found in our patient (table I) is in agreement with previous reports

(18, 48) The rate of appearance of Fe^{59} in erythrocytes was also found to be quite low (fig 2) with only 47% incorporation measured at the end of 14 days. A considerable degree of inefficient erythropoiesis, as described by FINCH (13) has to be assumed from these findings. It is most likely primarily due to intra marrow haemolysis.

Cellkinetic aspects The assumptions drawn from these ferrokinetic studies of an ineffective type of erythropoiesis playing a major rôle in the mechanism of anaemia in P A. is well in agreement with the concepts of the erythron as proposed by WEICKER (50 51) and LAJTHA (23) for this desease. WEICKER (51) postulated in 1955 that homoplastic divisions predominate in immature megaloblasts while homo-heteroplastic divisions are normally seen in this stage. He assumed that continued homoplastic reproduction of the stem cell cycle K_2 K_1 K_2 is the main reason for the excessive accumulation of immature erythrocytic forms in P A. While nuclear maturation is delayed and inhibited, the cytoplasmic development seems to proceed fairly normally Consequently K_2 and K_1 forms of higher than normal haemoglobin content may be found. These cells are characterized by nuclear-cytoplasmic asynchronism, a common finding in P A. In LAJTHA's (23) model, based on studies with cellular labels, prolongation of the second resting stage of intermitosis is postulated and a similar defect inhibiting cellular maturation

A number of methods have been used to investigate these cell kinetic relationships. Using the stathmokinetic method, ASTALDI (3) found higher indices for all megaloblastic forms compared with normal erythropoiesis. At the end of 18 hours of incubation, about 35% of basophilic and 23% of polychromatophilic megaloblasts were found to be blocked in metaphase by colchicine. These studies indicated that megaloblasts proliferate in the basophilic stage about twice as actively and in the polychromatophilic stage about four times as actively than normoblasts. On the other hand, ASTALDI (3) noted that mitotic indices were fairly similar in both series, while those reported by ROHR (39) are somewhat higher for basophilic megaloblasts than normoblasts. In our single case studied with H^3 tymidine (table II) we found a higher index of DNA synthesis only at the polychromatophilic stage (54% labeling) compared with findings in normoblastic erythropoiesis (23 % labeling of polychromatophils, 40)

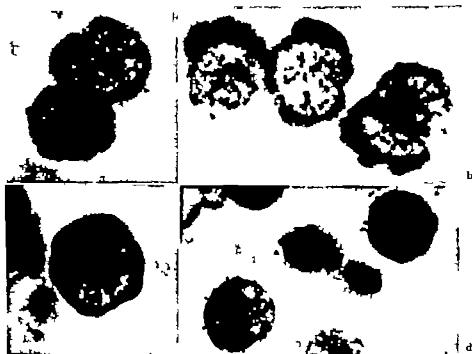


Fig. 7a–d. Photomicrographs (× 1600) of bone marrow autoradiograms illustrating basophilic megaloblasts labeled with a) H^3-thymidine, b) H^3-uridine, and c) d) H^3-cytidine. The incubation time used was 1 hour the concentration of the precursors in the incubation medium 0.8 μc/ml, the exposure time of the stripping film 8 days (7a) 19 days (7b) and 32 days (7c, 7d). Spec. Act. of all precursors 1.9 C/mM.

While conclusive figures about cycle times can only be obtained from in vivo studies or experiments with prolonged incubation time our data of the 1 hour in vitro DNA synthesis may still be valid to indicate an active proliferative potential of megaloblastic cell populations. Since several authors (3 4) have been able to ascertain that megaloblastic maturation time is markedly prolongated, the rapid cellular proliferation must be accounted for in P.A. by an increase primarily in homoplastic divisions, as indicated in WICKRAMASINGHE's (51) model Inhibition of nuclear maturation would therefore, be one of the basic pathogenetic principles in the anaemia of P A.

Studies of the in vitro RNA synthesis, using H^3 uridine and H^3-cytidine, revealed most active incorporation into basophilic megaloblasts and decreasing values for polychromatophilic and orthochromic forms In contrast to normal haemopoiesis (40) pro-

liferation and RNA synthesis apparently continue in P A. at the metamyelocyte stage. There is also some evidence of RNA synthesis occurring even in orthochromatic cells.

Biochemical aspects In vitro studies of marrow cultures from patients with P A have shown in the past that some biochemical defect other than inhibition of cell maturation alone must be present in this disease. ASTALDI (4) found that the megaloblastic cell cycle became accelerated by the addition of nicotinamide without resulting in normoblastic transformation On the other hand, normoblastic conversion was obtained by the addition of liver normal serum and folic acid (21 28 43) but not by vitamin B_{12}. To become metabolized, vitamin B_{12} apparently requires the presence of a binding factor (4 25 26) Such a factor has been identified in normal plasma (52)

Regarding the biochemical defect caused by vitamin B_{12} deficiency only hypotheses have been forwarded in the past. In 1958, REISNER (37) postulated for this disease an interference in DNA synthesis at the level of the methylation of deoxyuridylic acid to thymidylic acid. However subsequent studies showed that 5-methyl tetrahydrofolic acid (5-CH_3-FH_4) could not be the methyl donator in this step as REISNER (37) assumed, and REICHARD (36) and RABINOWITZ (34) presented evidence in 1960 that 5 10-methylen tetrahydrofolic acid (5 10-CH_2-FH_4) probably repre-

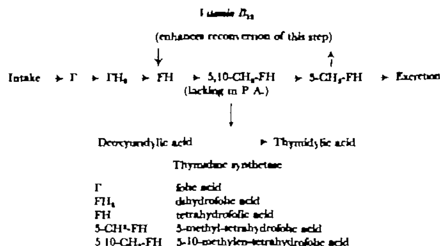


Fig. 8. Metabolic interrelationship of folic acid and vitamin B_{12} in the pathogenesis of megaloblastic haemopoiesis (49)

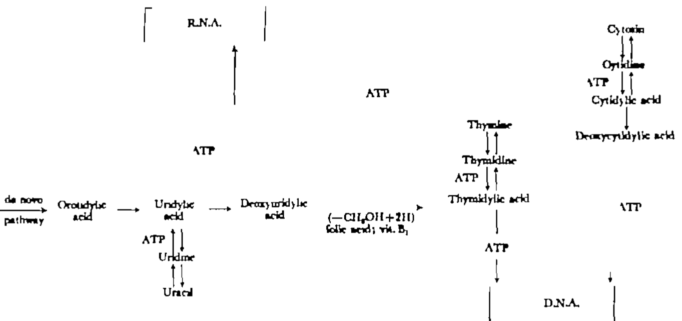


Fig. 9 Interconversion of pyrimidine nucleotides. Modified scheme of W.A. (47)

sents the coenzyme for the methylation of deoxyuridylic acid in several cell systems. Based on these findings, WATERS AND MOLLIN (49) concluded in 1963 that the reconversion of 5-CH_2-FH_4 to FH_4 could be blocked in P A. with resulting deficiency in 5 10-CH_2-FH_4. This substance would be required for synthesis of thymidylic acid from deoxyuridylic acid (fig 8)

Our studies indicate that radiouridine and radiocytidine are incorporated at a high rate in immature megaloblasts. As seen from fig 9 this finding reflects primarily intensive RNA synthesis. In spite of the inhibited synthesis of thymidylic acid from deoxyuridylic acid RNA synthesis continues to be extremely active in these cells. The morphological expression of active protein synthesis in these megaloblasts has to be related to the proliferative activity to haemoglobin synthesis and to cytoplasmic maturation. WILLIAMS et al. (54) found recently that the addition of B_{12} to P A. marrow cells in vitro stimulated the incorporation of radiocytidine into DNA bases but not into RNA bases. Our findings are along the same lines and would tend to support the concept that RNA synthesis proceeds normally in megaloblasts while DNA synthesis may be inhibited

Correlation of findings An attempt to correlate recent biochemical concepts with WEICKER s (51) and LAJTHA s (22) model of the erythron in P A. and erythrokinetic as well as autoradiographic findings may give a more unified picture about the pathogenetic mechanism of this disorder

It is known that human marrow cells rely almost entirely on the liver for the supply of purines (1 2 24) From the observed in vitro conversion of megaloblasts to normoblasts by the addition of certain substances, it has appeared likely in the past that a rather late step in nucleic acid synthesis was affected in P A. which normally has to proceed at least partially within the marrow cells themselves. The high rate of incorporation of radiouridine and -cytidine (RNA) into megaloblasts is a particularly important finding in this regard since inhibition of thymidylic acid synthesis from deoxyuridylic acid a step subsequent to the precursor used, has been postulated in P A. It may be an expression for cytoplasmic maturation proceeding fairly normally and more independent of B_{12} than nuclear maturation, or for the predominantly homoplastic divisions of the K_2-K_1 K_2 stem cell cycle (51) Release of inhibited thymidylic acid synthesis by therapy is promptly followed by

cellular maturation. Apparently a change to homoheteroplastic divisions occurs, establishing normoblastic erythropoiesis.

From these considerations it may be conceivable to assume that the aberrant biochemical pathway in P A. affects only DNA synthesis, while RNA precursors are normally utilized While cytoplasmic maturation appears to be primarily dependent upon RNA synthesis, impairment of DNA synthesis at the suggested step probably inhibits nuclear maturation. The aberrant nucleic acid pathway leading to megaloblastosis would lead to an inefficient type of erythropoiesis with intra marrow haemolysis and shortened survival of circulating erythrocytes as well. This inefficient erythropoiesis would be compensated by excessive proliferation of immature cells which are unable to mature normally due to defective DNA synthesis. RNA synthesis, on the other hand, seems to be not or less affected by B_{12} deficiency

Summary

Pathogenetic principles of pernicious anaemia are studied by labeling marrow cells of patient in vitro with DNA and predominant RNA precursors. Results with H -thymidine confirm that megaloblasts proliferate intensively in the basophilic and polychromatophilic stages. High rates of incorporation was also obtained for H^3-uridine and H^3-cytidine, especially in immature cells. In pernicious anaemia the aberrant megaloblastic erythropoiesis apparently utilizes uridine and cytidine abundantly in spite of the metabolic block postulated for thymidylic acid synthesis. This study supports previous findings that RNA synthesis is active and not affected by B_{12} deficiency Homoplastic cell divisions and inhibition of nuclear maturation seems to be characteristic features of megaloblastosis, leading clinically to severe anaemia and increased erythrocyte haemolysis. Red cell destruction apparently occurs partially within the marrow with inefficient erythropoiesis and low rate of Fe^{59} incorporation and partially in the peripheral blood with shortened survival and increased red cell iron turnover rate.

Résumé

La pathogénie de l'anémie pernicieuse est étudiée en marquant in vitro des cellules de la moelle osseuse à l'aide de précurseurs de l'ADN et de l'ARN Les résultats obtenus avec H -thymidine confirment une prolifération active des mégaloblastes basophiles et polychromatophiles. Une étude à l aide de H^3-uridine et H -cytidine montre également un taux d'incorporation important de ces substances particulièrement dans les cellules immatures. *A propos de l'anémie pernicieuse, l'érythropoïèse mégaloblastique* aberrante utilise apparemment une grande quantité d'uridine et de cytidine, malgré l'hypothèse d'un bloc métabolique concernant la synthèse de l'acide thymidique. Cette expérience confirme les découvertes précédentes qui suggèrent que la synthèse de l'ARN est active et non affectée par une déficience en vitamin B_{12} Une des caractéristiques de la mégaloblastose semble être représentée par des divisions cellulaires homoplastiques et une inhibition de la maturation nucléaire qui conduisent toutes deux à une anémie sévère et à une augmentation de l'hémolyse érythrocytaire La destruction des globules rouges se fait apparemment, d'une part dans la moelle, et d'autre part dans le sang périphérique. Dans la moelle cette destruction accompagne d'une production insuffisante d érythrocytes et d'une diminution du taux d'incorporation du Fe^{59} tandis

que dans le sang périphérique, on constate une diminution du temps de survie des globules rouges ainsi qu'une augmentation du «turnover rate» du fer globulaire.

Zusammenfassung

Zur Untersuchung der Pathogenese der perniziösen Anämie wurden Knochenmarkzellen mit DNS- und vorwiegend RNS-Vorstufen in vitro markiert. Mit H^3-Thymidin konnte festgestellt werden, daß Megaloblasten im basophilen und polychromatischen Stadium aktiv proliferieren. Eine hohe Einbau-Rate wurde in diesen Zellen auch für H^3-Uridin und H^3-Cytidin gefunden. Bei B_{12}-Mangel wurden trotz der aberrierenden megaloblastischen Erythropoese Uridin und Cytidin offensichtlich in normaler Weise verwertet, obschon ein Block in der Synthese der Thymidinsäure angenommen wird. Die Ergebnisse bestätigen, daß die RNS-Synthese im Knochenmark durch den B_{12}-Mangel nicht wesentlich beeinflußt wird. Vermehrte homoplastische Teilungen und eine gehemmte Kernreifung, welche als charakteristisch für die Perniziosa angesehen werden, stehen deshalb wahrscheinlich in Beziehung mit einer gehemmten DNS-Synthese. Ihre klinischen Manifestationen sind die schwere Anämie und der beschleunigte Erythrozytenabbau. Der letztere spielt sich teilweise innerhalb des Knochenmarkes ab mit resultierender frustraner Erythropoese und vermindertem Einbau von Fe^{59} in die Erythrozyten, teilweise aber auch im peripheren Blut mit verkürzter Überlebenszeit und gesteigertem Eisenumsatz der Erythrozyten.

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Attempt at Classification of Mouse Leukemia Viruses

Mouse Leukemia Virus Type A and the Friend Virus

By Ludwik Gross

In a preceding paper (3) we discussed the fact that the term "Mouse Leukemia" refers to a limited variety of forms of this disease observed in mice to develop spontaneously or induced by X rays or carcinogenic chemicals the most common form is lymphatic, essentially a generalized lymphosarcoma, and comparable to visceral lymphomatosis in the chicken (5) This disease is caused in mice by a virus, which we suggested (3) to designate as the Mouse Leukemia Virus Type A The many recent isolations of presumably distinct leukemogenic viruses either from spontaneous leukemia, or transplanted mouse tumors, and in certain instances also from radiation-induced leukemia may represent isolations from different sources of the same virus, or at best some of its close variants* According to this concept the mouse leukemia virus type A would cause the development of the common forms of mouse leukemia, such as lymphatic, stem-cell or myeloid leukemia, etc., as well as lymphosarcomas, or reticulum-cell sarcomas. Although there may exist some minor immunological differences between some of the variant strains of the mouse leukemia virus type A, isolated from different sources, these virus strains have the same physical and pathogenic properties, and they induce in mice (and rats) the same disease they represent basically the same virus (3)

Aided, in part, by grants from the Damon Runyon Memorial Fund and the American Cancer Society

* For more detailed information, and pertinent references, the interested reader is referred to the preceding paper of the author published in Acta haemat. (3) and also to the monograph on "Oncogenic Viruses" (5)

The Friend Virus

A different form of disease of the hematopoietic system which also belongs to the broad group of leukemias, is caused by a virus originally isolated by Dr CHARLOTTE FRIEND. This curious disease seems to be exceedingly rare under natural life conditions; it can be however readily induced by inoculating susceptible mice with a virus, originally isolated by FRIEND from a Swiss mouse (1, 2). Following inoculation of filtrates prepared from spleens and livers of donor mice with FRIEND-virus-induced leukemia, into 3 to 4 weeks old mice of several susceptible strains, such as DBA/2, BALB/c, C3H(f) or Swiss, progressive enlargement of spleen and liver is induced after a relatively short latency of a few weeks only; the disease may become chronic, with enlargement of spleen persisting over prolonged periods of time; very occasionally spontaneous regressions have also been observed[1]; in most instances, however, the disease is progressing rapidly and the animals die usually within 2 to 3 months after inoculation. The latency and survival time depend on the genetic susceptibility of the inoculated mice, and also on age at inoculation, and dose of the injected virus. The optimum time for inoculation is when mice od susceptible strains, such as C3H(f) or BALB/c, are about 3 weeks old. Injection of the filtrate into newborn or suckling mice of such strains induces a rapidly progressing disease of short duration.

The results are essentially the same, following inoculation of either filtrates, or cell suspensions prepared from spleens and livers of donor mice with FRIEND-virus-induced disease.

In our studies, carried out with either the Friend virus[2] or with the RAUSCHER virus strain[2], we have prepared filtrates from spleens and livers of the leukemic mice employing our method of preparation of leukemic filtrates previously described (4). Only filtrates were inoculated in our experiments. In most instances we injected i. p. 0.3 to 0.4 ml, each, of 5% filtrate into 3 to 3½ weeks old mice of either C3H(f) or BALB/c strains; at that age mice of both strains were found to be uniformly susceptible to either the FRIEND virus, or to the Rauscher virus strain.

In certain instances, mice of strain C57 Brown/cd, relatively resistant to the induction of the Friend disease, were also employed for inoculation.

In another series of experiments, newborn Sprague Dawley rats were employed for inoculation of either the FRIEND virus or of the RAUSCHER virus strain.

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[1] GROSS, L. Unpublished experiments (1963).

[2] We are grateful to Dr CHARLOTTE FRIEND for her virus received on December 14, 1962, from her laboratory at the *Sloan-Kettering Institute* in *New York City*. We also appreciate the receipt from Dr FRANK J. RAUSCHER of his virus strain on November 15, 1962, from the National Cancer Institute, Bethesda, Md.

Rats were found to be resistant to the development of the Friend form of disease, but developed lymphatic, stem-cell, or myelogenous leukemia following inoculation of either virus strain

In mice, the enlargement of spleen and liver is the main macroscopic picture of the induced disease there are no thymic tumors and *the peripheral lymph nodes are not enlarged. This is in striking* contrast to the great majority of mouse leukemias. On microscopic examination there is considerable destruction of the parenchymal liver cells, as well as infiltration of liver spleen and other organs with abnormal white blood cells. The peripheral blood shows the presence of a variety of cells of the myeloid series, large numbers of characteristic smudge cells, and many nucleated red cells. There is *also a rapidly progressing anemia.*

The Friend disease most probably belongs to the broad group of leukemias. In fact, it would be difficult to designate this condition by any other name. It is, nevertheless, very different from any of the usual forms of leukemia occurring spontaneously in mice and other animals species.

The Friend disease has no apparent relation to the presence or absence of thymus. Following inoculation of the Friend virus into susceptible mice, the course of the disease induced in thymectomized mice was the same as it was in non-thymectomized controls[2]

Development of Lymphatic Leukemia Following Inoculation of the Friend Virus

Under certain experimental conditions inoculation of the Friend virus may result in the development of lymphatic leukemia (fig 1) This may occur when the Friend virus is inoculated into mice which are relatively resistant to the Friend virus, such as mice of strain C57 Brown/cd (table I) or in rats[1] It would be possible to explain this by assuming that the Friend virus is capable of inducing either the Friend disease, or any other form of leukemia such as lymphatic or stem-cell leukemia i. e forms induced usually by the mouse leukemia virus type A (3) On the other hand, it is also possible to assume that what is now considered to be the Friend virus, may actually be a mixture of two distinct leukemic viruses.

Gross, L. Unpublished experiments (1963—1964) Also Mirand and Grace (7)

Gross, L. and Woolley, G W Unpublished experiments (1959) Also Metcalf et al. (6)

It is conceivable, that a filtrate prepared from spleens and livers of mouse donors with FRIEND-virus-induced disease may contain two distinct leukemic viruses, the FRIEND virus, and also the mouse leukemia virus type A. Following inoculation of such a filtrate into susceptible animals, either form of the disease could be induced. However the FRIEND virus induces disease after a relatively short latency and for that reason when susceptible mice are inoculated, they usually develop and die from, the FRIEND form of disease, before they have an opportunity to develop lymphatic leukemia, or another form of leukemia caused by the mouse leukemia virus type A.

Table I

Results of inoculation of FRIEND virus into C57 Brown mice

Strain	Age at inoc. days	No. of mice inoc.	No. devel. leukemia Type A	F	Aver. latency months	No. of surviv. mice	[illegible]
C57BR	< 8	17	8[2]	7	8	2	8
C57BR	< 28	4	3	1	8		
		21	11	8	8	2	8
Controls C3H(f)	< 28	20	0	19	< 1	1	< 2

Type A, usually lymphatic or stem cell, with thymic tumours, peripheral lymph nodes enlargement, etc., similar to that induced with passage A virus.

Type F (FRIEND) hepato-spleno-megaly with no thymic tumors, and no peripheral lymph node enlargement. Characteristic blood morphology showing presence of variety of white cells of the myeloid series, large number of smudge cells and many nucleated red cells also severe anemia.

2 mixed type, A and F

The Virus Strain Isolated by Rauscher

The leukemic virus strain isolated recently by RAUSCHER (9) appears to be very similar to if not identical with, the FRIEND virus following inoculation into either suckling, or 3 to 4 weeks old mice of several strains, such as C3H(f) or BALB/c, it induces a disease which is indistinguishable from that resulting from inoculation of the FRIEND virus. In this respect, the RAUSCHER virus strain is very similar to the virus recently isolated from wild mice in Australia by POPE (8)

The RAUSCHER virus strain appears to be also antigenically identical with the FRIEND virus. The FRIEND virus is substantially more antigenic than the mouse leukemia virus type A. It is possible to obtain an immune rabbit serum (2) which will neutralize the Friend virus in vitro in dilutions up to 1 10 11. have obtained such an immune serum by injecting rabbits at weekly intervals with FRIEND virus filtrates. Serum obtained



Fig. 1. Lymphatic leukemia induced in C57 Brown mouse with the FRIEND virus. This C57Brown/cd female mouse was inoculated (0.2 to 0.3 ip.) when less than 3 days old, with FRIEND virus (5% filtrate). After latency of 11 months, this mouse developed typical generalized lymphatic leukemia. Note large spleen, enlarged liver, large thymic and mesenteric tumors, and very large inguinal, axillary and cervical lymph nodes. Microscopic examination of tissues and blood smears revealed lymphatic leukemia. (Two additional mice in the same litter inoculated simultaneously with the same filtrate, also developed lymphatic leukemia.)

Table II

Attempt to neutralize the FRIEND virus by specific (rabbit) immune serum

Serum	No. of mice inoc.	No. dev. leuk.	Leuk. incid.	Aver. latency months	No. of remain. mice	Average age, remain. mice surv. months
Friend	34	11	32	5	23	6
Pass. A	14	14	100	1½		
Normal Mouse Organs	12	9	75	1	3[d]	
Controls: Virus only	36	36	100	1		

Serum obtained from rabbits that had received several injections, at weekly intervals, of either) FRIEND virus filtrate, b) passage A leukemic filtrate, or) filtrate prepared from normal C3H(f) mouse organs. All sera inactivated 56 °C for hour.

FRIEND virus filtrate, conc. 1 to 2 mixed 1 1 with serum diluted 1 2 to 1 10.

Mice of C3H(f) strain inoculated at 21 days of age.

17 of these died, or were sacrificed an average age of 6 months with no signs of leukemia; 4 still alive and well at 6 months of age.

All 3 alive and well at 6 months of age.

It is conceivable, that a filtrate prepared from spleens and livers of mouse donors with FRIEND-virus-induced disease may contain two distinct leukemic viruses the FRIEND virus, and also the mouse leukemia virus type A. Following inoculation of such a filtrate into susceptible animals, either form of the disease could be induced. However the FRIEND virus induces disease after a relatively short latency and for that reason when susceptible mice are inoculated, they usually develop and die from the FRIEND form of disease, *before they have an opportunity to develop lymphatic leukemia*, or another form of leukemia caused by the mouse leukemia virus type A

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Serum obtained from rabbits that had received several injections, at weekly intervals, of either a) Friend virus filtrate, b) passage A leukemic filtrate, or c) filtrate prepared from normal C3H(f) mouse organs. All sera inactivated at 56 °C for ½ hour. Friend virus filtrate, conc. 1 to 2%, mixed 1 : 1 with serum diluted 1 : 2 to 1 : 10.

Mice of C3H(f) strain inoculated at 21 days of age.

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[illegible] dem [illegible] die [illegible] der [illegible] die [illegible] Leukämie-Virus [illegible]

[illegible]

1. [illegible] C. [illegible] of a [illegible] for [illegible] of a leukemia [illegible]
2. [illegible] C. [illegible] of a [illegible] a leukemia in adult mice. J. [illegible]
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Gross, L.: Oncogenic Viruses, pp. [illegible] Pergamon Press, Oxford/New York [illegible]
5. [illegible] J. [illegible] of [illegible] leukemia [illegible] by Friend virus. Cancer Res. [illegible]
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8. [illegible] J. [illegible] of a mouse leukemia virus resembling Friend virus. [illegible] J. [illegible]
9. [illegible] A [illegible] of mice characterized by [illegible] J. nat. Cancer Inst. [illegible]

[illegible] New [illegible]

Acta haemat. 32, 89–99 (1964).

Aus dem Institut für normale und pathologische Physiologie der Universität Köln

Über die Entstehung von Aggregaten in ACD Blutkonserven

Von H. P. Künzel und H. Hirsch

Im ACD-Konservenblut kommt es während der Lagerung zu zahlreichen Veränderungen, wie Zunahme des Erythrozytenvolumens (18 25) Verlangsamung der Erythrozytensedimentierung (24) Erhöhung der Viskosität (16) Zunahme der Wasserstoffionenkonzentration und des extrazellulären Kaliums (22) Zunahme der Ammonium- Phosphat Laktat und Pyruvatkonzentration (22) und Zunahme des Plasmahämoglobins (22) Außerdem bilden sich Aggregate, die vorwiegend aus Thrombozyten bestehen (26) Über die Zunahme dieser Aggregate im Verlauf der Lagerung der Blutkonserven und die weitere Vermehrung dieser Aggregate durch Erwärmen der Blutkonserven wird hier berichtet.

Methodik

Zur Messung der Aggregate wurde die von Swank (26) beschriebene Apparatur verwendet, bei der eine bestimmte Menge Blut mit konstanter Geschwindigkeit durch ein Mikrosieb gepreßt wird. Der vor dem Sieb entstehende Druck – Siebungsdruck genannt – gibt eine quantitative Aussage über das Ausmaß der gebildeten Aggregate.

Zur Messung des Siebungsdrucks wird der Konus einer 5-ml-Glasspritze, die mit 5 ml Blut luftblasenfrei gefüllt ist, in eine entsprechende Bohrung des Plastikblocks (Abb. 1) eingeführt. Durch eine hydraulische Vorrichtung werden in 10 sec 2 ml Blut aus der Spritze durch den polierten Kanal (Ø 0,75 mm) im Plastikblock gedrückt. Der Kanal wird durch ein Mikrosieb, das von einer durchbohrten Gegenschraube fixiert wird, abgeschlossen. Der Druck, der vor dem Sieb entsteht, wird über einen seitlich vor dem Sieb abzweigenden Kanal mit einem Statham Strain Gauge Transducer über ein Hellige-Elektromanometer gemessen und mit einem Linienschreiber registriert. Die Mikrosiebe sind runde Plättchen von etwa 0,7 mm Durchmesser aus Folien (Buckbee-Mears-Co., St. Paul, Minnesota) mit 20 × 20 µ großen Löchern. Für jede Messung wird ein neues mit Ultraschall gereinigtes Mikrosieb verwendet. Spritzenauflagerung und Plastikblock werden auf 39 °C geheizt, damit die Proben bei annähernd 37 °C gemessen werden. Vor jeder Blutmessung wird mit demselben Sieb der Siebungsdruck

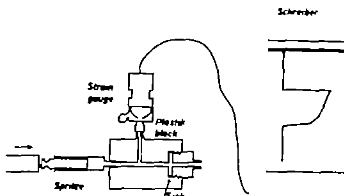


Abb. 1 Schema der Methode zur Messung des Siebungsdrucks (26)

Wegen der Begrenzung des oberen Meßbereiches von benutzter Meßbrücke und Statham Transducer wurden nur Siebungsdrucke bis 600 mm Hg gemessen. Waren die Siebungsdrucke hoher als 600 mm Hg, wurde die Messung bei Erreichen von 600 mm Hg unterbrochen.

Bei allen Messungen wurden nur die amerikanischen 5-ml-Multifit-Glasspritzen benutzt, da nur sie in ihren äußeren Abmessungen so standardisiert sind, daß vergleichbare Messungen möglich sind.

Die Konservenflaschen waren Einmalflaschen des Biotest-Serum-Instituts, Frankfurt/Main (100 ml Bioflaschen mit 20 ml ACD-Stabilisator) und der Firma Braun, Melsungen (500 ml Einmalflaschen mit 100 ml ACD-Stabilisator).

Der Hämatokrit wurde nach der Methode von Wintrobe (29) ermittelt.

Die Blutproben wurden aus den Konserven nach Umschütteln durch sterile Punktion entnommen. Die Entnahmespritzen wurden mit einer Kappe verschlossen und vor der Messung 3 min in ein Wasserbad von 37 °C gehängt.

Ergebnisse

1 Veränderung des Siebungsdruckes von Blutkonserven während der Lagerung bei 4 C Von 11 Blutkonserven wurde der Siebungsdruck zu verschiedenen Zeiten während der Lagerung bei 4 C bestimmt. Die Konserven wurden 16 bis 22 Tage gelagert. Die Spender waren männliche gesunde Personen im Alter zwischen 20 und 30 Jahren. Bei 7 Konserven wurde die erste Bestimmung 15 bis 30 min nach Abnahme gemacht. Dann erfolgte bis zum 16 Tag jeden 2 Tag eine Kontrolle des Siebungsdrucks. Bei 2 Konserven wurde der Siebungsdruck zum erstenmal am 2. Tag, dann fortlaufend jeden 2. Tag bis zum 22 Tag gemessen Bei 2 Konserven wurde der Siebungsdruck nur am 8., 12 15 und 19 Tag bestimmt.

Die Messungen (Abb 2) zeigen, daß der Siebungsdruck 15 bis 30 min nach der Konservenfüllung 13 bis 20 mm Hg beträgt. Die



Abb. 2. Siebungsdrucke von 11 ACD-Konserven während der Lagerung

Siebungsdruckkurve steigt nach einem Anfangsausschlag nicht weiter an (Abb. 3). Der plateauförmige Kurvenverlauf entspricht dem, der bei der Messung von Ringerlösung registriert wurde. Das Plateau liegt bei Ringerlösung nur tiefer wegen der niedrigeren Viskosität. Der plateauförmige Kurvenverlauf zeigt, daß praktisch keine Poren des Mikrosiebes verstopft werden, daß also noch keine Aggregate vorliegen. Der niedrige Siebungsdruck des frisch entnommenen Konservenblutes ist von der Viskosität abhängig. Am 2. Tage war der Siebungsdruck schon bei einem Teil der Proben erhöht: eine Konserve hatte schon einen Siebungsdruck von 146 mm Hg (Abb. 2). Der Siebungsdruck dieser Konserve hatte bereits am 4. Tage 600 mm Hg überschritten. Diese hohen Siebungsdrucke erreichten noch zwei weitere Konserven: eine am 10. und eine am 16. Tage. Während der Lagerung stieg der Siebungsdruck aller Konserven mehr oder weniger stark, fast kontinuierlich an. Am Ende der Kontrollzeit war der Siebungsdruck von nur 3 der 11 Konserven noch unter 50 mm Hg. Die Druckkurven von ACD-Blut, das Aggregate enthält, zeigen einen anderen Verlauf (Abb. 3). Der Druck steigt während der ganzen Meßzeit immer weiter an.

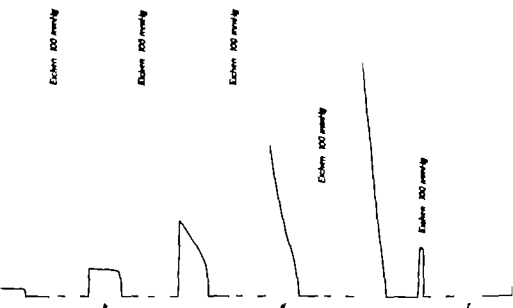


Abb. 3 Kurven der Siebungsdruckmessung von Ringerlösung und einer ACD-Konserve während der Lagerung a = Ringerlösung, b = 30 min nach Konservenfüllung, c = 2 Tage nach Konservenfüllung, d = 4 Tage nach Konservenfüllung, e = 8 Tage nach Konservenfüllung, f = 10 Tage nach Konservenfüllung. Die Druckkurven sind von rechts nach links zu lesen.

Jetzt ist der Siebungsdruck von Zahl und Größe der Aggregate abhängig.

Bei der ersten Messung jeder Konserve wurde der Hämatokrit bestimmt. Er lag zwischen 40 bis 50 Vol.%. Zwischen ihm und der Höhe des Siebungsdrucks fand sich keine Beziehung.

2. *Veränderung des Siebungsdrucks von gelagerten Blutkonserven nach Änderung der Umgebungstemperatur von 4° auf 23° C.* Nach der Lagerung bei 4° C, die 16 bis 22 Tage dauerte, wurden die 11 Konserven einer Temperatur von 23° C ausgesetzt. Die erste Bestimmung des Siebungsdrucks wurde direkt nach Herausnahme der Konserve aus dem Kühlraum gemacht. Dann wurde die Konserve in ein Wasserbad von 23° C gesetzt, der Siebungsdruck wurde stündlich gemessen. Abb. 4 zeigt das Verhalten des Siebungsdruckes bei 23° C, der Anfangspunkt der Kurven ist der Siebungsdruck direkt nach Entnahme aus der Blutbank. Die Abb. 4 ist die Fortsetzung der Abb. 2. Die 3 Konserven, die schon bei der Lagerung einen

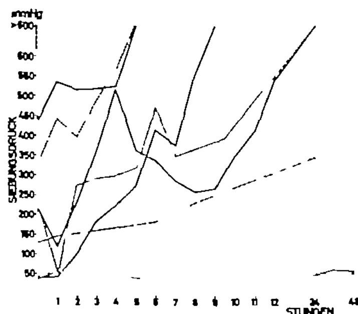


Abb. 4. Siebungsdrucke von 8 gelagerten ACD-Konserven nach Änderung der Temperatur von 4 auf 23 °C. In der Abb. sind die Siebungsdrucke der Konserven, die schon während der Lagerung über 600 mm Hg betrugen, nicht eingezeichnet. Die Abb. ist eine Fortsetzung der Abb. 2; die Konserven sind mit denselben Symbolen bezeichnet wie in Abb. 2.

Siebungsdruck von über 600 mm Hg erreicht hatten, und nicht mehr in der Abb. 4 dargestellt. Ihr Siebungsdruck stieg bei 23 °C weiter an, war jedoch nicht mehr quantitativ zu messen. Im Verlaufe von Stunden stieg der Siebungsdruck bei den übrigen 8 Konserven bis auf 2 schnell an. Bei diesen 2 Konserven handelt es sich um diejenigen, deren Siebungsdruck bei der Lagerung nur auf 41 bzw. 37 mm Hg angestiegen war. Ihr Siebungsdruck erreichte nach 24 Std. bei 23 °C nur 55 bzw. 60 mm Hg.

Bei 90 Konserven wurde der Siebungsdruck 1 bis 3 Std. nach der Transfusion bestimmt. In der Regel waren diese Konserven in der Klinik ohne Aufwärmen bis auf 30 bis 50 ml transfundiert worden. Der Siebungsdruck dieses restlichen Blutes war bei 73 Konserven höher als 600 mm Hg, d.h. es lagen in über 80 % Aggregate in einer solchen Menge vor, daß sie mit dieser Methode nicht mehr quantitativ meßbar waren. Die Konserven waren 4 bis 28 Tage alt, die jüngste Konserve, die schon einen Siebungsdruck von über 600 mm Hg hatte, war erst 4 Tage alt.

In Blutausstrichen, die nach PAPPENHEIM gefärbt wurden, fanden sich neben frei liegenden Erythrozyten zahlreiche einzelne Thrombozyten und ab und zu umfangreiche, verschieden große Anhäufungen von Thrombozyten in lockerer Verklumpung. Die Einzelstruktur der aggregierten Plättchen war zum Teil noch erkennbar. In manchen, vorwiegend größeren Aggregaten waren auch Leucozyten enthalten.

Diskussion

Der Siebungsdruck von Blutkonserven während der Lagerung. In ACD-Konserven entstehen während der Lagerung Aggregate. Die *Aggregate bestehen vorwiegend aus zusammengeballten Thrombozyten.* In größeren Aggregaten waren teilweise auch Leucozyten enthalten. Diese Befunde bestätigen die früher von SWANK (26) erhobenen.

Eine quantitative Aussage über die Größe und Anzahl der Aggregate erlaubt der Siebungsdruck. Dieser steigt im Verlauf der Lagerung an. Am Abnahmetag liegen noch keine Aggregate vor, der Siebungsdruck beträgt dann 13 bis 20 mm Hg. Während der Lagerung kommt es zu einer zunehmenden Bildung von Aggregaten, so daß der Siebungsdruck stetig ansteigt. Der Grad des Anstiegs ist aber von Konserve zu Konserve verschieden. In manchen Fällen ist er nur schwach ausgeprägt. Unsere Ergebnisse bestätigen damit frühere Untersuchungen von SWANK (26).

Für die relativ große Schwankung des Siebungsdrucks bei *gleich alten Konserven verschiedener Spender sind* wahrscheinlich mehrere Gründe verantwortlich. So schwankt die Zahl der Thrombozyten individuell erheblich (6). Der Gesundheitszustand scheint ferner die Zahl der Aggregate zu beeinflussen: bei Infektionen und postoperativ war sie erhöht (17). Auch bei Thrombosen und Hyperkoagulabilität des Blutes war die Adhäsionsneigung der Thrombozyten gesteigert (28).

Normalerweise wird, wie die Erfahrung der Klinik zeigt, ACD-Konservenblut trotz des im allgemeinen hohen Siebungsdrucks vom Empfänger ohne auffallende Störungen vertragen. Bei schon erniedrigter Strömungsgeschwindigkeit in einzelnen Organen kann es jedoch beim Empfänger leichter zu Störungen kommen. Auch wird die transfundierte Menge und die Art der Applikation – venöse oder arterielle – von Bedeutung sein. Bei der venösen Transfusion

wird ein großer Teil der Aggregate in der Lunge abgefangen. Bei arterieller Gabe sind eher Störungen denkbar So konnten wir zeigen, daß es bei der Perfusion des isolierten Katzenkopfes mit arteriellem Heparinblut, das einen hohen Siebungsdruck hatte, zu erheblichen Störungen, ja sogar zum Erlöschen des ECoG kommen konnte (7) Es ist also möglich, daß beim Vorliegen einer Kreislaufinsuffizienz oder eines Schocks, wo Blutdruck und Blutvolumen herabgesetzt sind, oder beim Vorliegen einer thromboembolischen Erkrankung wo die Fibrinolyse herabgesetzt ist, bei Transfusionen größerer Mengen von Blut, das viel Aggregate enthält, der Zustand des Patienten durch weitere Erniedrigung der Strömungsgeschwindigkeit eher verschlimmert werden kann. Es sollte so gefährdeten Patienten möglichst Frischblut gegeben werden, oder falls dies nicht möglich ist, zuerst ein Plasmaexpander bis eine gewisse Normalisierung der Strömungsgeschwindigkeit erreicht wird. Erst dann sollte eine Konserve transfundiert werden, die fast immer Aggregate enthalten wird. Die Konserve muß nach den »Richtlinien für die Einrichtung des Blutspendewesens« der Deutschen Gesellschaft für Bluttransfusion (20) wenigstens 72 Std. alt sein, um eine Lueinfektion sicher auszuschließen wird die Konserve eher gebraucht, so sind besondere Vorsichtsmaßregeln einzuhalten.

Der Einfluß der Temperaturänderung auf den Siebungsdruck nach Herausnahme aus der Blutbank. Nach Erhöhung der Umgebungstemperatur stieg der Siebungsdruck weiter aber ungleich schneller an. Nur bei 2 der von uns angelegten Konserven stieg der Siebungsdruck nicht wesentlich an. Die in der Klinik transfundierten Konserven, die 1 bis 3 Stunden nach der Transfusion gemessen worden waren, die also eine längere Zeit bei Zimmertemperatur gehalten worden waren, zeigten in einem höheren Prozentsatz als die von uns angelegten Konserven Siebungsdrucke von über 600 mm Hg Die Ursache hierfür ist eine andere Spenderauswahl Das Blut der von uns angelegten Konserven stammte von gesunden Jugendlichen, die noch nie vorher gespendet hatten.

Die Erhöhung des Siebungsdrucks bei Zimmertemperatur unterstützt die Empfehlung Blutkonserven möglichst bald nach Entnahme aus der Blutbank, also noch kalt zu transfundieren. Nicht statthaft ist das Aufwärmen in einem Wasserbad mit höherer Temperatur (5, 19 20 23) Eine Hypothermie des Patienten ist nur bei massiven und schnellen Transfusionen zu befürchten (2) In solchen Fällen wird die Einschaltung eines Durchlauferwärmers in

das Transfusionsbesteck empfohlen, dessen Wassermantel nicht über 37 C sein darf (2)

Bisher sind zwei einfache Methoden bekannt, mit denen die Aggregate abgefangen und durch die der hohe Siebungsdruck normalisiert werden kann, ohne das Blut sonst nennenswert zu verändern. Experimentell erprobt sind Filter aus Pyrex-Glaswolle (11 26) Dabei ist jedoch die Gefahr gegeben, daß Glaswollstückchen in das Blut gelangen und zu Gefäßspasmen führen. Diese Gefahr besteht nicht bei Verwendung mehrerer Lagen von Nylongewebe (11 26)

Thrombozytenadhäsivität und Thrombozytenaggregation. Über die Ursache der Aggregatbildung in ACD-Konservenblut sind bisher keine verbindlichen Aussagen möglich. Bei der Messung des Siebungsdrucks wird außer der Aggregation von Thrombozyten, die auch als «unspecific clumping» (12) oder als Agglomeration (13) bezeichnet wird, wahrscheinlich auch die Thrombozytenadhäsivität, d.h. die Fähigkeit, an fremden Oberflächen haften zu können, gemessen. Bei allen Methoden, die nur die Adhäsivität erfassen, wird die Verminderung der Thrombozytenzahl nach Kontakt mit Glas (bzw Glaswolle) gemessen (6 14 15 21 30) Diese Adhäsivität ist abhängig von der Art des verwendeten Antikoagulans (6) von der Temperatur (6) und dem Hämatokrit (13 9 6) Wahrscheinlich ist zwischen Adhäsivität und Aggregation kein genereller sondern nur ein gradueller Unterschied zu machen. Stärkere Adhäsivität führt zu Aggregation. Hellem (6) vermutete, daß durch Oberflächenkontakt ein Stoff aus den Erythrozyten frei wird – Faktor R genannt – der Thrombozyten zur Adhäsion und bei höherer Konzentration zur Aggregation bringt. Der Faktor R soll *Adenosindiphosphat sein* (4)

Hohe Siebungsdrucke werden außer bei gelagertem ACD- und Heparin Konservenblut (26) bei starkem Blutdruckabfall im hämorrhagischen Schock (27) und bei starker Histaminhypotonie (10) in vivo gefunden. In vitro entstanden Thrombozytenaggregate durch Einwirkung von $HgCl_2$, $HgCN_2$ und Saponin (17) Serotonin (8) und Adenosindiphosphat (1 4) Auch das Antikoagulans Heparin läßt Aggregate entstehen (3 6 12 26)

Zusammenfassung

Bei mit ACD stabilisiertem Konservenblut wurde der Siebungsdruck im Verlauf der Lagerung bei 4 °C mit der Methode von Swank gemessen. Er gibt eine quantitative Aussage über Anzahl und Größe von Aggregaten, die vorwiegend aus Thrombocyten

und Leukocyten bestehen. 30 min nach Füllung der Blutkonserve beträgt der Siebungsdruck unter 20 mm Hg. Im Verlauf der Lagerung steigt er in unterschiedlichem Maße an (schon am 2. bzw. 4. Tage im Einzelfall auf 146 bzw. über 600 mm Hg). Wird die Blutkonserve vor der Transfusion bei Zimmertemperatur aufgewärmt, so steigt der Siebungsdruck weiter an, meistens ungleich schneller.

Auf die möglichen Auswirkungen beim Empfänger durch Transfusion einer größeren aggregatreichen Blutmenge wird hingewiesen. Es wird empfohlen, Konserven nur dann zu verwenden, wenn eine ausreichende Strömungsgeschwindigkeit vorhanden ist. Eine erheblich verminderte Strömungsgeschwindigkeit sollte vor der Transfusion von Konserven durch Plasmaexpander angenähert normalisiert werden. Es wird weiter empfohlen, die Konserven unaufgewärmt zu transfundieren und die Aggregate durch Filter vor der Transfusion weitgehendst zu entfernen.

Summary

The screen filtration pressure was measured in ACD bank blood during storage at 4 °C with the method of SWANK. It is quantitatively related to the amount of aggregates which consist mostly of thrombocytes and leucocytes. In freshly prepared bank blood the screen filtration pressure is initially less than 20 mm Hg and increases during storage to various degrees (maximum values 146 on the 2nd and more than 600 mm Hg on the 4th day). The increase of the screen filtration pressure was accelerated, when bank blood was warmed up to room temperature before transfusion.

Some possible effects in recipient patient due to massive transfusion of aggregate-rich blood are discussed. It is suggested to use bank blood only when blood flow is normal or close to normal. The latter should be restored towards normal values by plasma expanders before transfusion of bank blood. In view of the possible presence of aggregates stored blood should be transfused without rewarming, and the aggregates should be removed by filters as completely as possible before transfusion.

Résumé

Les changements de la «screen filtration pressure» (pression obtenue en filtrant du sang au moyen de filtres à membrane sous conditions standardisées) ont été mesurés sur du sang conservé à 4 °C par addition du mélange ACD. La pression de «screen filtration» mesurée selon la méthode de SWANK est en relation quantitative avec le nombre et la grandeur d'aggrégats, formés surtout de thrombocytes et de leucocytes. La pression de «screen filtration» est inférieur à 20 mm Hg dans le sang de banque fraîchement préparé et augmente en cours de conservation (valeurs maximales 146 au deuxième jour et supérieure à 600 mm Hg au quatrième jour). L'augmentation de la pression est trouvée accélérée lorsque le sang a été ramené à la température ambiante avant la transfusion.

Les troubles au cours d'une transfusion massive de sang riche en aggrégats sont discutés. Il est suggéré de n'utiliser du sang de banque que lorsque le débit sanguin est normal ou proche de la normale. Le retour à la normale devrait être assuré avant la transfusion par «plasma expanders». Le sang de banque doit être transfusé sans réchauffement, et les aggrégats cellulaires doivent être éloignés par des filtres.

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The Place of the Lymphocyte in the Reticulo-endothelial System: A Study of the in vitro Effect of Prednisolone on Lymphocytes

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The development of a system of culture of human lymphocytes under the influence of phytohaemagglutinin (PHA) or some antigenic substances, has led to a better understanding of lymphocyte function. It has been found that the synthetic steroid prednisolone is a potent inhibitor of the action of PHA (16, 31) We have extended this line of investigation in order to discover the relationship between the time of steroid treatment and the inhibition of lymphocyte transformation and also to demonstrate an alteration of the sensitivity of lymphocytes to prednisolone by PHA. It is hoped that studies such as these may throw some light on the relationship of the transformed lymphocyte to the different types of cells found in the reticulo-endothelial system in vivo.

Methods

Lymphocytes were obtained from the blood of normal individuals and cultures set up as has been previously described (17) Phytohaemagglutinin (P Difco) was added to all cultures t given time. Prednisolone 21-phosphate ("Codelsol") was added to the cultures at various times in final concentration of 0.1 mg/ml of culture medium. After 72 hours all cultures were terminated and the cells removed by gentle centrifugation (500 rpm) for 5 minutes the cells were then resuspended in drop of cell-free normal human serum and spread on slides which were then stained with Jenner-Giemsa stain. Differential cell counts of 200 lymphoid cells were made for each culture. Transforming cells were identified by their larger size, lighter staining nuclei with prominent nucleolus, and increased basophilia of the cytoplasm. In order to determine absolute numbers of "lymphoid cells cell counts and differential counts were performed on cultures which were terminated after 24 hours.

Results

One of the most striking features of prednisolone-treated cultures was the good preservation of the neutrophil polymorphs. In

both treated and control cultures the eosinophils remained in good condition.

Table I

The effect of prednisolone on cell counts after 24 hours of culture.

Interval between addition of PHA and prednisolone	Absolute "lymphoid cell" count (cells/mm³)	
	Range	Mean
Prednisolone 3 hours before PHA	298–360	323
Prednisolone simultaneous with PHA	790–848	830
Prednisolone 5 minutes after PHA	726–880	792
Prednisolone 15 minutes after PHA	725–880	845
Prednisolone 1 hour after PHA	792–848	818
No PHA	280–342	309
No prednisolone	980–1047	1017

a) *Absolute numbers of lymphoid cells* As will be seen in table I there was a marked decrease in the number of lymphoid cells in cultures to which no PHA had been added or to which PHA had been added some time after the steroid. In cultures to which both prednisolone and PHA had been added simultaneously and in those to which the hormone had been added shortly after the PHA there was only a small fall of about 200 cells compared with the number of lymphocytes in cultures without prednisolone.

b) *The degree of lymphocyte transformation.* The figures are shown in table II. There was a very marked inhibition of transformation if prednisolone was added before the PHA, but it is worthy of note

Table II

Effect of prednisolone (0.1 mg/ml) on PHA treated cultures.

Interval between addition of PHA and prednisolone	Degree of lymphocyte transformation (%)	
	Range	Mean
Prednisolone 1 3 hours before PHA	6–13	9
Prednisolone added simultaneously with PHA	9–25	14
Prednisolone 5 minutes after PHA	27–47	34
Prednisolone 10 minutes after PHA	40–54	46
Prednisolone 15 minutes after PHA	41–62	51
Prednisolone 30 minutes after PHA	41–61	49
Prednisolone 45 minutes after PHA	42 70	52
Prednisolone 1 hour after PHA	45–60	52
Prednisolone 2 hours after PHA	42 58	52
Prednisolone 3 hours after PHA	41–60	51
Prednisolone 6 hours after PHA	47–62	53
Prednisolone 20 hours after PHA	46–68	53
Prednisolone 48 hours after PHA	45–67	54
Control	40–70	54

PHA culture without prednisolone.

that some lymphocytes were still able to transform. There was still a considerable degree of inhibition when the two substances were added simultaneously but after 5 minutes of PHA treatment the prednisolone was less potent as an inhibitor and by 10 minutes there was little or no difference between these cultures and the PHA treated control series. The rather low mean percentage seen in the 10-minute cultures was due to a low degree of transformation in one culture only the remaining cultures treated at this time showed little difference from the controls.

Discussion

In this culture system prednisolone clearly has a lethal effect on lymphocytes untreated with PHA. It has been well known for many years that conditions of stress and injection of adrenal cortical extract will produce a fall in blood lymphocyte numbers, and decrease in weight of the lymphoid tissue (11 12 41 42) The results of in vitro studies of the effect of adrenal steroids on lymphoid cells have been confusing some have found no effect using cortisone (3 34) whereas others find a marked lethal effect (35 40) HECHTER AND JOHNSON (26) found a toxic effect of adrenal cortical extract on lymphocytes after one hour of culture in some circumstances, but found cortisone to be harmless. These workers, like others, used only a short incubation period, and TROWELL (40) has since observed that the effect of these hormones is slow and increases logarithmically for a period of 24 hours. KRIPPHAENE AND OSGOOD (29) using a similar method to ours, utilising PHA, found no harmful effect of cortisone in these experiments however PHA was used as a separating agent and the cells had been exposed to it for some time before the cortisone was added.

Our results show that there are some normal small lymphocytes which are resistent to prednisolone at this dose and also that there are some cells which cannot be rendered resistant to the hormone by PHA. It may be suggested that some of the prednisolone-resistant cells may have already been immunologically stimulated in vivo because in lymphocyte cultures untreated with PHA, or any other antigen, we invariably find a small percentage of transformed cells. In all cultures treated with prednisolone at 0 hours or after there was a small reduction in the number of cells. In these cultures, however there were varying degrees of transformation which were

not significantly different from the controls after 5—10 minutes of prednisolone-free exposure to PHA. This must indicate firstly that some of the PHA cells are killed by the steroid as well as non transforming cells, and secondly that there is a graded effect by means of which the cells may be protected by the PHA and yet not transform if the stimulating phase is interrupted early on by the hormone (i. e. within 5–10 minutes).

This work is interesting in view of the studies by STEFANI AND SCHREK (36) who used similar cultures to demonstrate the ability of PHA to make lymphocytes radioresistant. Treatment of the intact animal with x irradiation results in a reduction of the antibody forming-capacity of the animal (9 22, 38) FITCH et al. (22) found that this failure to produce antibody was correlated with a depression of the formation of the large pyroninophilic cells in the spleen. In this context it has been found that the antibody producing system becomes radioresistant once the antigenic stimulus has been received although it is extremely radiosensitive before the antigen is administered (10 22, 28).

Adrenal cortical steroids have also been found to exert a profound effect on the antibody producing mechanism in vivo. Early reports seemed to indicate that adrenal cortical extract enhanced antibody levels in the blood (13) Other groups found that these steroids did not enhance antibody levels (15 23) or increase serum protein turnover (37) It is now clear that cortisone inhibits antibody formation (6, 21 23 25) and it has been shown by some workers that to be effective the cortisone must be given before the antigen (2 4 20) It is also possible however that the pattern of sensitivity to adrenal cortical hormones varies with the type of antigen (5 25) If the PHA transformation is an immune phenomenon as has been suggested (18) then the experiments reported above would indicate that the process of initiating changes in the cell which determine its antibody producing role is a rapid one and may correspond to the brief radioresistant "adaptation" phase discussed by DIXON et al. (10)

The histological picture seen in the lymphoid tissue after treatment with these hormones is essentially one of lymphoid atrophy with failure to develop germinal centres and less pronounced plasma cell formation (6 42) The cells of the active germinal centre are very similar to those produced from lymphocytes by PHA or antigenic stimulation they are large primitive

cells, with nucleoli and very basophilic cytoplasm. They have been shown to be capable of DNA synthesis (44) and to produce antibody (32) PHA cells have also been shown to synthesize DNA (8 30) and to produce antibody (18, 27) If these two cell types are identical, and the products of the antigenic stimulation of the lymphocyte, then it would seem that the lymphoid germinal centre is an antibody producing organ rather than a major site of lymphopoiesis as is generally supposed. To support this suggestion are the observations that germinal centres appear at the height of their development after the introduction of antigenic material (1 14 33) and their absence from lymphoid tissue of animals reared in an aseptic environment (24)

The PHA cell, in its resistance to irradiation, is similar to the plasma cell series, and some forms of reticulum cells, which it may also resemble morphologically although a clear association between them has not been demonstrated by electron microscope studies (19 39) It is also perhaps of significance that prednisolone is limited in therapeutic usefulness to those reticuloses in which the basic cell type is the small lymphocyte, and is almost ineffective against tumours containing reticulum cells (7 43)

Summary

Prednisolone added to lymphocyte cultures before or simultaneously with phytohaemagglutinin (PHA) produced significant inhibition of lymphocyte transformation It produced a small degree of inhibition if added 5 minutes after PHA but after 10 minutes and subsequently caused no inhibition. PHA had the effect of protecting the lymphocytes from the cytolytic action of prednisolone if added at the same time or before the steroid. The similarity between the effect of radiation and prednisolone in vitro is discussed and the action of the hormone on the cultured cells is compared with the in vivo effect of it on the lymphoid tissue and immune responses. The PHA cell in *its resistance to irradiation and adreno-cortical hormones is similar to the plasma cell* series and some reticulum cell types.

Résumé

L addition de prednisolone aux cultures d lymphocytes avant ou simultanément avec l'addition de phytohémagglutinine (PHA) provoque une inhibition significative de la transformation des lymphocytes. L'inhibition est faible, si l'addition est opérée 5 minutes après celle du PHA, elle disparaît totalement si l'addition est opérée 10 minutes ou plus tard après le PHA. Le PHA protège les lymphocytes contre la cytolyse de prednisolone 'Il est ajouté simultanément ou avant le stéroïde. Les auteurs discutent

les analogies entre l'effet d'une irradiation et de celle de la prednisolone in vitro et comparera l'influence hormonale sur la culture cellulaire avec l'effet sur le tissu lymphatique et les *réactions immunologiques in vivo. La cellule, influencée par le PHA, ressemble* par sa résistance contre l'irradiation et les hormones corticosurrénales aux plasmocytes et à quelques cellules réticulaires.

Zusammenfassung

Die Zugabe von Prednisolon zu Lymphocyten-Kulturen vor oder gleichzeitig mit Phytohämagglutinin (PHA) führte zu einer signifikanten Hemmung der Transformation von Lymphocyten. Erfolgte die Zugabe 5 Minuten nach PHA, so war die Hemmung nur geringgradig, erfolgte sie nach 10 Minuten oder noch später so blieb die Hemmung aus. PHA schützte die Lymphocyten vor der zytolytischen Wirkung von Prednisolon, wenn es gleichzeitig mit oder vor dem Steroid zugefügt wurde. Die Ähnlichkeit der Wirkungen von Bestrahlung und Prednisolon *in vitro* wird besprochen und die Hormonwirkung auf die Zellkultur wird verglichen mit dem Effekt auf lymphoides Gewebe und Immunreaktionen *in vivo*. Die unter dem Einfluß von PHA stehende Zelle gleicht in ihrer Resistenz gegen Bestrahlung und Nebennierenrindenhormone der Plasmazellreihe und einigen Reticulumzellformen.

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Zur Methodik und Spezifität des Glykogennachweises in den Leukozyten mittels der PAS-Reaktion

Von G. Mähr

Der Glykogennachweis erfolgt heutzutage in den Leukozyten praktisch nur mehr mittels der PAS-Reaktion, da deren chemischer Reaktionsablauf im Gegensatz zur früher angewandten Jod- und Carminfärbung genau bekannt ist. Zusätzliche Differenzierungsverfahren erlauben dann noch eine weitere Unterscheidung der dargestellten Substrate. Die fermentative Entfernung der PAS-positiven Substanzen aus den Zellen durch Diastase wird in der Hämatologie allgemein als beweisend für Glykogen angesehen (3, 5, 7, 8). Diskrepanzen zwischen quantitativen biochemischen und zytochemischen Glykogenbestimmungen in den Leukozyten von Diabetikern ließen mittlerweile die Frage diskutieren, ob der PAS-Reaktion in Verbindung mit der Diastaseprobe tatsächlich eine entsprechende Spezifität zur Darstellung von Glykogen zukommt (4). Außerdem stimmen die Angaben über die Diastase- und Hyaluronidasewirkung auf die Polysaccharide der einzelnen Leukozytenarten in der Literatur nicht überein.

Zum Nachweis der Spezifität der PAS-Färbung habe ich die für diese Färbung gebräuchlichen histochemischen Reaktionen zuzüglich der Diastase- und Hyaluronidaseproben angewandt. Da die Diskrepanzen in der Literatur möglicherweise auf Unterschiede der Methode beruhen, wurde gleichzeitig bei allen Färbungen durch Modifikation der einzelnen Reaktionen versucht, die für die Leukozyten geeignetsten Methoden zu finden.

Methodik*

Es wurden Blutausstriche von Gesunden den verschiedenen Färbemethoden unterworfen. Als Kontrolle wurde stets ein Ausstrich nach der einfachen PAS-Methode mitgefärbt.

1. *PAS-Färbung*

Die Färbung erfolgte in der üblichen Weise. Es zeigte sich bei zusätzlichen Untersuchungen der Fixierungsmöglichkeiten, daß Methyl- oder 96% Äthylalkohol durch 10 Minuten optimal sind. Längere Fixierungen (2–48 Stunden) in Äthylalkohol ergaben keine besseren Resultate. Fast etwas prägnanter wurden die Ergebnisse durch Formoldämpfe (5 Minuten).

Die Verseifung mit KOH bei der Desacetylierung führte fast regelmäßig trotz größter Vorsicht zur Ablösung der Blutfilme vom Objektträger. Auch die Vorbehandlung mit Celloidin änderte diese Ergebnisse nicht. Dies veranlaßte uns, die Fixierung nach Gendre zu versuchen. Tatsächlich gelang es damit, die Striche so weit zu erhalten, daß eine Beurteilung der Zellen möglich war. Der Gendreschen Fixierung wird von Romeis überhaupt der Vorzug zur Glykogendarstellung gegeben.

Zur Herstellung der Schiffschen Lösung hat sich das von Graumann (6) angegebene Pararosanilin (Merck 7601) am besten bewährt. Mit basischem Fuchsin nach den Originalangaben von Hotchkiss wurde die Färbung etwas schwächer. Schiffs Reagens Merck ist durch die Verwendung von Diamantfuchsin statt basischem Fuchsin bzw. Pararosanilin für diese Zwecke nicht geeignet.

2. *Spezifitätsnachweise*

a) *Nachweis von freien Aldehyden.* Durch direkte Färbung mit Schiffscher Lösung ohne vorherige Oxydation mit Perjodsäure erübrigt sich die Sulfitblockierung nach Lillie (11).

b) *Lipidextraktion.* (14) Die luftgetrockneten Ausstriche wurden wie histologische Schnitte 24 Stunden in Methylalkohol-Chloroform bei 60 °C gleichzeitig fixiert und inkubiert und anschließend mit 70% Alkohol gewaschen. Die anschließende Färbung mit Sudan-Schwarz B fiel komplett negativ aus, während die PAS-Reaktion positiv blieb.

c) *Acetylierungstest* (12). Die Ausstriche müssen nach der Fixierung sofort naß eingelegt werden, da sonst die Reaktion nicht abläuft. Die Leukozytenkerne blieben besser erhalten, wenn die Ausstriche ohne Abspülen mit Wasser direkt vom Alkohol in die Lösung gegeben wurden. Die nach Gendre fixierten Striche sind besser vorher mit Wasser zu spülen.

d) *Desacetylierung.* Wie schon anfangs erwähnt, gelang die Fixierung nur nach Gendre. Erst damit konnten Ergebnisse erzielt werden, die eine qualitative Beurteilung gestatten.

3. *Enzymtests*

a) *Speichel- und Diastaseproben.* 60 Minuten Inkubation in Speichel bei Laboratoriumstemperatur ergab eine völlig negative PAS-Reaktion in den alkohol- bzw. formol-fixierten Präparaten. Bei pflanzlicher (Merck) und tierischer (Fluka) Diastase

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* Die Untersuchungen wurden zur Kontrolle auch an Kryostatschnitten von Leber und Schilddrüse durchgeführt. Für die Überlassung der Schnitte und wertvolle Ratschläge sei Herrn OA. Dr. G. Benner und Frl. E. Klöss vom Path. Anat. Institut (Direktor: Prof. Dr. W. Sandritter) vielmals gedankt.

0,2% in physiologischer Kochsalzlösung war die Reaktion gelegentlich nicht vollständig. Es fand sich oft noch eine leichte diffuse Rötung des Plasmas.

Für den Speicheltest empfiehlt sich die übliche Fixierung, denn in den Carnoy fixierten Ausstrichen bewirkten sowohl Speichel als auch die Diastasen meist eine unvollständige Verdauung.

b) *Hyaluronidase* (1000 E. Kinetin, Schering/ml): 60 Minuten Inkubation verursachten eine geringe Abnahme der Rotfärbung. Dieselbe Minderung der Farbintensität ließ sich aber auch an Kontrollpräparaten mit Wasser beobachten.

Ergebnisse

Die gewonnenen Resultate sind in Tabelle I zusammengestellt. Die Ergebnisse der PAS-Reaktion in den Blutzellen sind hinreichend bekannt, so daß nicht näher darauf eingegangen werden soll.

Tabelle I

		Eosinophile		Basophile		
	Neutrophile	Ortopl.	Granula	Ortopl.	Granula	Lymphozyten
PAS	+	+	—	+	+	+
Freie Aldehyde	—	—		—	—	—
PAS nach Lipoidextraktion	+	+		+	+	+
Acetylierung	—	—		—	—	—
Desacetylierung	+	+		+	+	+
Diastase	—	±		—	—	—
Hyaluronidase	+	+		+	±	+

Ohne vorherige Oxydation fiel die Leukofuchsinreaktion in allen Zellen negativ aus. Freie Aldehyde sind daher in den Leukozyten nicht vorhanden. Nach der Lipoidextraktion war die PAS-Reaktion unverändert positiv. Glykolipoide sind somit an der PAS-Reaktion in den Leukozyten nicht beteiligt.

In allen Zellen ließen sich die freien Aldehyde nach Oxydation durch Acetylierung blockieren und anschließend durch KOH wieder verseifen. Das zugrunde liegende Substrat müssen demnach Alpha-Glykole bilden. Hyaluronidase führte lediglich in den Granula der Basophilen zu einer Reaktionsabschwächung, woraus nur für diese Zellen der Nachweis von sauren Mucopolysacchariden möglich ist. Den Befund von STORTI u. Mitarb. (17), daß Hyaluronidase zu einer negativen PAS-Reaktion in den Neutrophilen führt, können wir auf Grund unserer Beobachtungen nicht bestätigen. Wohl bekamen auch wir nach 15stündiger Inkubation in Hyaluronidase in den Neutrophilen eine negative Reaktion. Wurden aber die Ausstriche zur Kontrolle genau so lange in Wasser gelegt, waren alle Zellen mit Ausnahme der Lymphozyten anschließend ebenfalls

PAS-negativ Die Hyaluronidasewirkung ist demnach nur ein Pseudoeffekt. Jeder längere Kontakt mit einer wäßrigen Lösung scheint auch nach der Fixation zu einem vollständigen Glykogen schwund aus den Zellen zu führen. Außerdem geht aus dieser Beobachtung hervor daß das Lymphozytenglykogen in einer wesentlich schwerer löslichen Form vorliegt.

Was die Diastasereaktion anlangt, fanden wir wie HECKNER (8) im Gegensatz zu ASTALDI (1) daß die Basophilengranula trotz ihres Gehaltes an sauren Mucopolysacchariden negativ werden. Die zytoplasmatische Grundsubstanz der Eosinophilen färbte sich dagegen nach Diastasewirkung nur mehr schwach an, was im Widerspruch zu den Erfahrungen HECKNERS (8) steht. Nach unseren Untersuchungen müßte der Großteil der Polysaccharide in den Eosinophilen ebenfalls aus Glykogen zusammengesetzt sein.



Abb. 1 Granula in nach GENDRE fixierten Eosinophilen.

Einen auffälligen Befund konnten wir bei der Fixierung nach GENDRE erheben Es fanden sich nämlich in den meisten Eosinophilen mehrere sehr grobe Granula (Abb 1) die keinerlei weitere zytochemische Reaktionen gaben. Diese Granula sind wahrscheinlich als Artefakte anzusehen. Eine sichere Abklärung ist uns jedoch nicht gelungen, so daß Art und Bedeutung dieser Veränderungen noch offen gelassen werden müssen. Die Interpretation der verschiedenen Resultate stoßt immer wieder auf Schwierigkeiten, und es ist HECKNER (7) nur beizupflichten daß sämtliche Verfahren der Kohlenhydratzytochemie selbst bei sorgfältiger Technik keines-

wegs zu immer identischen und reproduzierbaren Ergebnissen an ein und demselben Objekt führen.

Zusammenfassung

Nach Erprobung mehrerer Modifikationen der in der Histochemie üblichen Untersuchungen zur PAS-Reaktion werden die für die Leukozyten geeigneten Methoden angegeben. Dabei gelingt die Verseifungsreaktion praktisch nur an nach Gendre fixierten Ausstrichen. Abweichend vom üblichen Bild der PAS-Färbung treten dabei in den Eosinophilen grobe Granula auf, die auf Grund des Fehlens jeglicher weiterer chemischer Reaktionen als Artefakte gedeutet werden. Die Gesamtheit der zytochemischen Reaktionen spricht dafür, daß der PAS-Reaktion in den Leukozyten, mit Ausnahme der Basophilengranula, in der Hauptsache Glykogen zugrunde liegt. Der nach biochemischen Untersuchungen in den Leukozyten gefundene Mucopolysaccharidanteil, der etwa die Hälfte der Polysaccharide beträgt (13), kommt zytochemisch nicht zur Darstellung. Lediglich in den Eosinophilen scheint ein Teil der zytochemisch nachweisbaren Polysaccharide als Muco- oder Glykoproteide vorzuliegen.

Summary

After various modifications of the usual histochemical methods for investigating the PAS reaction had been tried, the methods best suited to the leukocytes are indicated. The saponification reaction can practically only be carried out on smears fixed according to Gendre. In contrast to the usual appearance of PAS staining, coarse granules are then seen in the eosinophils. In the absence of any other chemical reactions, these must be regarded as artefacts. All the cytochemical reactions indicate that glycogen is chiefly responsible for the PAS reaction in the leukocytes, with the exception of the basophilic granules. The mucopolysaccharide portion found in the leukocytes on biochemical examination, corresponding to about half the polysaccharides present (13), is not demonstrated cytochemically. Only in the eosinophils does a proportion of the polysaccharides which can be shown cytochemically appear to be in the form of either mucoproteins or glycoproteins.

Résumé

Après essai de plusieurs modifications de la réaction histochimique de PAS, usuelle dans l'histochimie, l'auteur indique la modification la mieux appropriée aux leucocytes. La saponification ne réussit pratiquement qu'après la fixation selon Gendre. En opposition aux résultats habituels de la réaction de PAS, les éosinophiles contiennent après le traitement selon le procédé de l'auteur des granulations plus grandes, qui, faute d'autres réactions chimiques, doivent être désignées comme artificielles. L'ensemble des réactions cytochimiques semble démontrer que la réaction de PAS est due chez les leucocytes, surtout au glycogène, à l'exception des granulations basophiles. Les mucopolysaccharides des leucocytes, déterminées par des réactions biochimiques, qui constituent environ la moitié des polysaccharides leucocytaires, ne sont pas mises en évidence par des méthodes cytochimiques. C'est seulement une partie des polysaccharides des éosinophiles, démontrées par méthode cytochimique, qui est constituée par des muco- ou glycoprotéides.

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Acta haemat. 32, 114–126 (1964)

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A Cytological and Cytochemical Study of Chediak's Leukocytic Anomaly*

By C. Mauri and V. Silingardi

The main cytological feature of the syndrome first described by Béguez César (3), Steinbrinck (23), Chediak (7) and Higashi (10) is the presence of anomalous giant granulations in all types of leucocytes and in reticulum cells. A careful description of the morphology of granules and of their staining properties with conventional blood cells staining mixtures was given by several authors (4, 5, 7, 8, 9, 17, 20, 25, 27). An electron microscope investigation was recently carried out by Bessis et al. (5). In spite of these investigations, some cytological questions are still open to discussion, in the first place the nature of the mononuclear cells containing the large azurophilic inclusion bodies.

Also the chemical composition of Chediak's inclusion bodies is still little known. As first observed by Higashi (10), the giant granulations in neutrophils, eosinophils, monocytes, and reticulum cells are strongly peroxidase positive (2, 5, 9, 10, 17, 20, 25, 27) while azurophilic corpuscles in lymphocytes and plasma cells are peroxidase negative (5, 9, 20, 27). No type of giant granules is stained with the Feulgen test (2, 20, 27). Other cytochemical findings are discussed. Among them, the presence and the meaning of the P.A.S. positivity and of sudanophilia of the giant granules in the different cell types are the chief unsolved questions.

Materials and Methods

A cytological and cytochemical study was performed on blood smears, spleen imprints, and bone marrow smears of a child affected by a typical Chediak's anomaly studied in Brazil by De Barros and Ribeiro de Barros (8). Dr. De Barros kindly provided us with the material.

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* This investigation was supported by a research grant from the Consiglio Nazionale delle Ricerche, Roma.

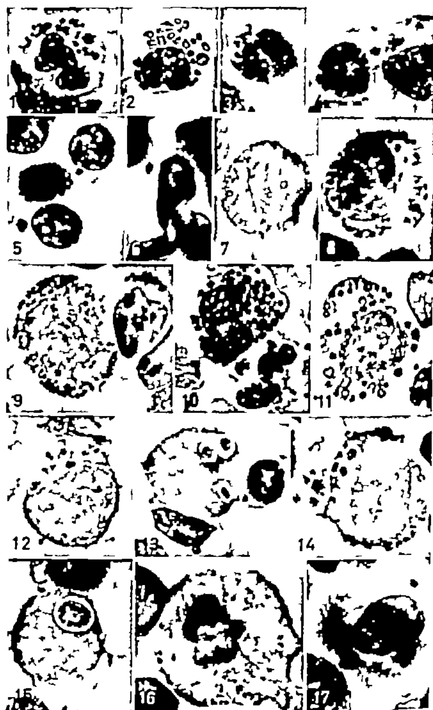
Since few smears were available only fundamental cytochemical reactions for lipids, polysaccharides, proteins, and nucleic acids were carried out. The staining methods used for the detection of these substances are listed in *table I*. Techniques were applied after PEARSE (18) No enzymatic study was done, since smears were about two years old. Reaction for alkaline phosphatase according to KABA A F TH, was negative and the search for peroxidases, according to S TO (21), gave incomplete and very weak positive findings in the giant granules of neutrophils, eosinophils, monocytes and reticulum cells, but not in lymphocytes.

Results

a) Cytological observations Large mononuclear cells containing one or more round, purple-stained bodies, sometimes surrounded by a pale zone, were present in bone marrow and spleen imprints (fig 12–16) Their morphological features point to a reticulum rather than to a myeloid cell In fact the size of these cells is larger than that of myeloblasts and promyelocytes Nuclear chromatin pattern resembles that of reticulum cells Cytoplasmic granulations are always azurophilic. No specific neutrophilic or eosinophilic granules are seen in these cells. Not only the large purple inclusion bodies (fig 13–15) but also the small azurophilic granulations which are spread in the cytoplasm, are often surrounded by a small pale vacuole-like zone (fig 16) Large purple bodies with pale non homogeneous central zone are rare in reticulum mononuclears (fig 15) Atypic azurophilic granules of CHEDIAK s true myeloblasts, on the contrary often contain a central pale area (ring granules) (fig 7–9) Several granules sometimes tend to join in larger azurophilic bodies. We agree therefore with the opinion of UNDRITZ (9 27) that the large mononuclears are reticulum cells and not immature myeloid cells, as stated by many authors (7 8 10, 11 17 20) They differ also from macrophages, whose cytoplasmic inclusion bodies are quite different (fig 17)

Although similar the pattern of purple inclusion bodies is different in large mononuclear cells, and in lymphocytes. In *lymphocytes* the inclusion bodies are always few in number small or middle sized, deeply and homogeneously azurophilic (fig 5 6) A pale vacuole like zone, surrounding the purple bodies, is quite exceptional in lymphocytes (fig 6)

DOEHLE like zones present in cytoplasm of *neutrophilic poly nuclears* are not be interpreted as true DOEHLE bodies (3) as seen in neutrophils in some infections diseases DOEHLE bodies are clearly although weakly basophilic, and their outlines are shaded. CHE


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DIAK's DOEHLE-like bodies have on the contrary sharply-cut out lines with spiny edges, and stain greyish or greyish brown (fig 1)

Our observations on the giant granulations of other leukocytes and blood forming cells, as *eosinophils* (fig 2 10 11) *basophils* (fig 3 4) and *plasma cells* are in agreement with the descriptions of other authors (4 5 9 17 20 27) *Erythroblasts megakaryocytes* and *blood platelets* are always normal.

b) Cytochemistry Our cytochemical findings are listed in *table I*

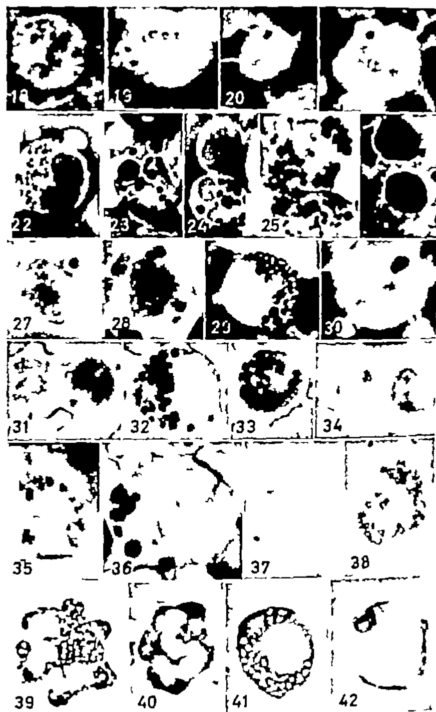
Desoxyribonucleic acid. Anomalous giant granules lack DNA, since the FEULGEN reaction appears negative in all granulation types, as described by other authors (2, 20 27)

Ribonucleic acid. With the methyl green/pyronine mixture the DOEHLE-like bodies of neutrophilic polynuclears (fig 18) and the inclusion bodies of lymphocytes (fig 20) and reticulum cells (fig 21) stain red. Normaly shaped eosinophilic granules appeared unstained, but large eosinophilic granules and crystal like eosinophilic bodies took up a weak pink stain (fig 19) Pyronine staining was negative after treatment with ribonuclease. On the contrary completely negative findings in all cells have been described by ASTALDI AND STROSSELLI (2) and by PAGE et al. (17) Also Gallocyanin test for nucleic acids has been found to be negative (2)

Proteins The presence of proteins was ascertained by the acid fast green staining method (pH 1.2) and by the ninhydrin/SCHIFF test in all anomalous granulations. DOEHLE like bodies of neutrophils (fig 22) were strongly but not homogeneously stained. DOEHLE like zone appears sometimes built up by two or more small round, cluster-arranged, positively stained bodies. Stain strength was higher in normal-shaped eosinophilic granules (fig 25) than in large crystal-like bodies (fig 23 24) With acid fast green staining large inclusion bodies of lymphocytes (fig 24 26) and reticulum cells (fig 27 28) exceptionally appeared located in a distinct vacuole.

Also some of the tests of D NELLI for proteins are positive in the giant granulations (2). The presence of protein-linked SH groups in reticulum cells inclusion bodies is stated by TERNER (25) but denied by ASTALDI A STROSSELLI (2) This question

Fig 1–17 Morphology of CHEDIAK *leukocytic anomaly (*MAY-GRUNWALD-GIEMSA *stain)* 1) Neutrophilic granulocyte with DOEHLE-like bodies 2) Eosinophilic granulocyte with crystal-like bodies. 3–4) Basophilic granulocytes with giant granules. 5–6) Lymphocytes with inclusion bodies. 7 9) Neutrophilic promyelocytes with ring or giant azurophilic or specific granules. 10–11) Eosinophilic myelocytes with large round granules. 12 16) Reticulum cells with several types of azurophilic inclusion bodies. 17) Macrophage.


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ought to be re-examined, since the cytoplasm of all normal blood cells and the specific leukocytic granules (except in basophils, 16) contain protein-linked SH groups, which react with Mazia's (15) and DDD (6) methods.

Lipids Döhle-like bodies of neutrophilic polynuclears were sudanophilic but no strong staining was observed Nile blue gave acid reaction U V /Schiff reaction was faintly positive. Plasmal test gave doubtful results. Baker's test (fig 31) copper phtalocyanin method and Okamoto's test were positive, but weaker than in normal cells. Only moderate quantities of phospholipids (acid esterphosphatides) are, therefore present in Döhle-like bodies of Chédiak's neutrophils.

Normally shaped eosinophilic granules and crystal-like bodies were strongly sudanophilic, mostly at the level of the cortex of the bodies, while the centre appeared sudanophobic (fig 29) Similar findings were also obtained with copper phthalocyanin (fig 38) Nile blue stained some eosinophilic bodies pink and others blue, even in the same cell. Baker's test (fig 32, 33) appeared strongly positive in the whole eosinophilic body Plasmal test, performic acid/Schiff and U V /Schiff reactions were negative or uncertain. Okamoto's staining procedures gave a weak reaction. Many crystal like bodies were almost negative and contained one or more small, round or rod-shaped positive localizations in the middle of the body (fig 37)

Fig 18–42. Cytochemistry of Chédiak *leukocytic anomaly*

Fig 18–21 Pyronin/methyl green. 18) Neutrophilic granulocyte with Döhle-like bodies. 19) Eosinophilic metamyelocyte with crystal-like bodies. 20) Lymphocyte with inclusion body 21) Reticulum cell with inclusion body

Fig 22–28. Acid fast green (pH 1 2) 22) Neutrophilic metamyelocyte with Döhle-like bodies. 23–24) Eosinophilic granulocytes with crystal-like bodies (in 24 also lymphocyte with inclusion body) 25) Eosinophilic myelocyte with large round granules. 26) Lymphocyte with inclusion body 27–28) Reticulum cells with large inclusion bodies.

Fig 29–30 Sudan black B 29) Eosinophilic myelocyte with crystal-like bodies. 30) Reticulum cell with large inclusion body

Fig 31–36. Baker *acid haematein* 31) Neutrophilic granulocyte with Döhle-like bodies and lymphocyte with inclusion body 32) Eosinophilic myelocyte with crystal-like bodies. 33) Eosinophilic granulocytes with large granules 34) Lymphocytes with inclusion bodies. 35–36) Reticulum cells with many inclusion bodies.

Fig 37 Okamoto *mercury diphenylcarbazone method.* eosinophilic myelocyte with crystal-like bodies.

Fig 38. Copper phthalocyanin method. eosinophilic myelocyte with round granules

Fig 39–42. Periodic acid/Schiff 39) Neutrophilic granulocyte with weakly positive Döhle-like bodies, strongly positive granules and vacuoles. 40) Neutrophilic granulocyte with positive Döhle-like bodies. 41) Eosinophilic myelocyte with negative crystal-like bodies. 42) Reticulum cell with strongly positive inclusion body

Table 1 Cytochemical findings in Che… anomalous cytoplasmic inclusions

Fixatives	Extraction or blocking procedures	Reactions	Azurophilic inclusion bodies: Lymphocytes	Azurophilic inclusion bodies: Reticulum cells	Double-blue bodies	Blue round eosinophilic granules	Eosinophilic crystal-like granules
Formalin 10% pH 7 10 min		Feulgen	0	0	0	0	0
Carnoy 5 min		Pyronin/methyl green	++	++	++	±	±
Carnoy 5 min	Ribonuclease (1 mg/ml and 5 mg/ml) 1 h, 37 and 60 C	Pyronin/methyl green	0	0	0	0	0
Formalin 10 pH 7 10 min		Fast Green pH 8.2 (22)	+	++	++	++	+
Formalin 10% pH 7 10 min		Ninhydrin/Schiff	+	+	+	+	+
Formalin vapour 10 min		Sudan black B	0	++	+	++	++
Formal vapour 10 min	Acetic acid 25 2 min	Sudan black B	0	+/0	±/0	++	++
Formol calcium 6 hrs		Nile blue	0	bl	blue	blue/pink	blue/pink
Formol calcium 6 hrs		Acid haematein (Baker)	+	++	+	++	++
Weak sol Bouin fixat 8 hrs	Baker pyridine extraction test	Acid haematein (Baker)	0	0	0	±/0	±/0
$HgCl_2$ 1 5 min		Plasmal test (Feulgen and Voit)	0	±	?	0	0
Formalin vapour 10 min		U V/Schiff	±	±	+	?	?
Formalin vapour 10 min		Performic acid/Schiff	0	±/0	0	0	0
Formalin vapour 10 min		Okamoto mercury diphenylcarbazone (U na)	0	+	+	+	+
Formalin vapour 10 ml		Copper phthalocyanin (ethanol)	0	+	±	+	+
Methanol 5 min		Periodic acid/Schiff (Hotchkiss-McManus)	+	++	+/±	+/0	+/0
Methanol 5 min	Saliva 30 ml 37 °C	Periodic acid/Schiff (Hotchkiss-McManus)	0	±	0	+/0	+/0
Methanol 5 min	Diastase (10 mg/ml) 30 min, 37 °C	Periodic acid/Schiff (Hotchkiss-McManus)	0	±	0	+/0	+/0
Methanol 5 min	H_2O 30 min, 37 °C	Periodic acid/Schiff (Hotchkiss-McManus)	+	++	+/±	+/0	+/0
Weak sol. Bouin fixat. 8 hrs		Periodic acid/Schiff (Hotchkiss-McManus)	+	++	+/±	+/0	+/0
Weak sol Bouin fixat. 8 hrs	Baker pyridine extraction test	Periodic acid/Schiff (Hotchkiss-McManus)	+	++	+/±	+/0	+/0
Weak sol Bouin fixat. 8 hrs	Bromination 3 hrs	Periodic acid/Schiff (Hotchkiss-McManus)	+	++	+/±	+/0	+/0
None		Aqueous toluidine blue (0,1 mg/ml) 30 min	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
None	[illegible] 30 [illegible]	Aqueous toluidine blue 0,1 [illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
None		[illegible]					

It is presumable that more types of lipids, of acid and non acid type, are mixed or stratified in the large eosinophilic granules and crystal-like bodies Phospholipids are the main component.

No sudanophilic bodies were seen in lymphocytes, even after unmasking treatment with acetic acid, according to ACKERMAN (1) On the contrary a positive reaction was observed in some lymphocytes with the BAKER's test (fig 31 34) After pyridine extraction BAKER's staining appeared negative. A faintly pink staining of the inclusion bodies was sometimes seen with U V /SCHIFF Other staining methods for lipids gave no reliable results. The presence of phospholipids in the cytoplasmic inclusion bodies of lymphocytes, stated by ASTALDI (2) is therefore presumable however this important question will be the subject of further investigation, when fresher material will be available.

Strongly positive findings for lipids were, on the contrary observed in the inclusion bodies of reticulum cells. Bodies were sudanophilic (fig 30) gave acid (blue) reaction with Nile blue, became stained greyish blue with the OKAMOTO's test and stained green with the copper phthalocyanin test. BAKER's test was strongly positive (fig 35 36) and U V /SCHIFF and plasmal tests gave a faintly pink stain. The presence of phospholipids has been, therefore, ascertained. Acid esterphosphatides and traces of acetalphosphatides are presumably present.

Sudanophilia and BAKER's test positivity were described by ASTALDI (2) in all anomalous granulations, but SARAIVA et al. (20) deny the sudanophilia of lymphocytic granules. Sudanophilia of the inclusion bodies of reticulum cells was ascertained also by TERNER (25)

Polysaccharides. In neutrophilic polynuclears many small strongly P A. S. positive granules were present, spread in the cytoplasm (fig 39) while DOEHLE-like bodies stained only pale pink (fig 39 40) Sometimes negative vacuoles were also seen (fig 39) Previous treatment with pyridine or bromination did not modify the strength of the colouring. On the contrary saliva or takadiastase prevented almost completely the positivity of the P A.S. reaction in DOEHLE like bodies. Toluidine blue stained ortochromatic. The presence of small quantities of glycogen in DOEHLE-like bodies was therefore demonstrated.

Normally shaped eosinophilic granulations were P A.S -negative, while the surrounding cytoplasm stained faintly pink. Large

eosinophilic granules and crystal like eosinophilic bodies were either negative or positive, although red stain was never strong (fig 41) P A. S positive and negative bodies might be present in the same cell. Takadiastase and saliva did not abolish the P.A.S positivity of the eosinophilic granules. No metachromasia was seen with toluidine blue. A polysaccharide (probably a non acid mucopolysaccharide) may therefore be present in some anomalous eosinophilic granules. No glycogen is here demonstrable.

Giant granules of reticulum cells (fig 42) and lymphocytes gave positive reaction with the periodic acid/SCHIFF test. Previous extraction with pyridine or bromination did not modify the strength of the colouring The P.A.S positivity is, therefore, not to be attributed to the presence of unsaturated ethylene binding in lipids. After treatment with takadiastase or saliva, the P A.S reaction was very weakly positive in the giant bodies of reticulum cells and fully negative in lymphocytes. No metachromatic stain was seen with toluidine blue at pH 6 nor metachromasia was induced by sulphuric acid esterification. P A.S positivity appears, therefore, mainly due to the presence of glycogen both in reticulum cells and in lymphocytes. The peripheric vacuole-like structure of giant bodies was P A.S. negative.

The P A.S positivity of all anomalous granulations was observed also by ASTALDI AND STROSSELLI (2) and in reticulum cells by TERNER (25) On the contrary negative findings were obtained by PAGE (17) as well as by SARAIVA et al. (20) except in lymphocytes, where the reaction was prevented by previous treatment with saliva. ASTALDI AND STROSSELLI (2) deny that the P.A.S. positive localizations are related to the presence of polysaccharydes, since no modification of the reaction was obtained with the previous treatment with saliva takadiastase, hyaluronidase, and acetylation according to LILLIE. It is presumable that the age of the blood and bone marrow smears available to ASTALDI, has been of some influence on the results of the hydrolysis procedures.

Peroxidase test. As mentioned above, the material available was about two years old. Some positive stain was nevertheless observed in all types of giant granules, except in lymphocytes.

Conclusions

The presence of giant specific granules in myeloid cells and of large azurophilic inclusion bodies in reticular and lymphoid cells

are the most important morphological features of Chediak's leukocytic anomaly Light and electron microscope observations show that in myeloid cells a pathological formative process of specific granules takes place in early maturation stages by fusion of several granules. Myelinic figures have been observed in Doehle-like bodies and in crystal like structures of eosinophils. On the contrary in the inclusion bodies of monocytes and lymphocytes a greyish matrix with dark granules was seen at the electron microscope. No common morphological structure is so far apparent in the many types of Chediak's giant granules.

Also cytochemical findings are different in each granulation type. Inclusion bodies in lymphocytes appear rich in proteins, RNA, polysaccharides (mainly glycogen) A phospholipid component is presumable in some granules, but the question needs further investigation. The large inclusion bodies of reticulum cells contain a great amount of proteins, RNA, polysaccharides (mainly glycogen) and various kinds of lipids (acid esterphosphatides and acetalphosphatides) Doehle like bodies in neutrophilic poly nuclears contain lipids (acid esterphosphatides) in smaller quantity than normal granules, and only traces of polysaccharides but a large amount of proteins and RNA. In eosinophilic polynuclears, the large round granules show a cytochemical pattern similar to normally shaped granules. Large amounts of proteins and of acid and non acid (phospho)lipids (mainly in the cortex of the granules) and small amounts of RNA are found. Usually polysaccharides are not present in granules, although present in the remaining cytoplasm however some eosinophilic granules may contain a P.A.S positive substance as in leukaemia and other pathological conditions (19 24) The large eosinophilic crystal-like bodies always contain larger amounts of polysaccharides, RNA and lipids and presumably fewer proteins than normal granules. No giant granule contains DNA. Except in lymphocytes and plasma cells, all abnormal granules are strongly peroxidase-positive.

The chemical composition of the giant granules of the myeloid cells appears, therefore qualitatively but not quantitatively close to normal. On the contrary inclusion bodies in reticulum and lymphoid cells are unlike any normal granulation of these cell types, and unlike any known storage process, including Alder's leucocytic anomaly (14) and classical lipidoses (12, 13)

Since each type of pathological granule and inclusion body has a different cytochemical pattern, multiple metabolic deviation would seem to take place in CHEDIAK's leukocytic anomaly Ribonucleoproteins, polysaccharides and (phospho)lipids storage are probably the main features.

No cytochemical anomaly was observed in erythroblasts, megakaryocytes, and blood platelets.

Summary

A cytological and cytochemical study in case of CHEDIAK's leucocytic anomaly was carried out. Large basophilic mononuclears, containing the characteristic azurophilic inclusion bodies, were considered as reticulum rather than immature myeloid cells. Cytochemical findings are different in each granulation type. The chemical composition of the giant granules of the myeloid cells appears qualitatively but not quantitatively close to normal. Inclusion bodies in reticulum and lymphoid cells are chemically unlike any normal granulation of these cell types, and unlike any known storage process. Storage of ribonucleoproteins, polysaccharides, and phospholipids appears to be the main feature.

Zusammenfassung

Bei einem Fall von CHEDIAK'scher Leukocytenanomalie wurden cytologische und cytochemische Untersuchungen vorgenommen. Die großen Mononukleären, die die charakteristischen azurophilen Einschlußkörper enthalten, wurden nicht als unreife myeloide Zellen, sondern als Reticulumzellen betrachtet. Die cytochemischen Ergebnisse waren verschieden je nach der Art der pathologischen Granulation. Die chemische Zusammensetzung der Riesengranula der myeloiden Zellen scheint qualitativ nicht aber quantitativ nahezu normal zu sein. Die Einschlußkörperchen der Reticulumzellen und der Lymphocyten unterscheiden sich chemisch von den normalen Granula dieser Zellen. Eine Speicherung von Ribonukleoproteinen, Polysacchariden und Phospholipiden dürfte die wichtigste Veränderung darstellen.

Résumé

Les auteurs procèdent à des études cytologiques et cytochimiques d'un cas de l'anomalie leucocytaire de CHEDIAK. Les grandes cellules mononucléées, contenant les inclusions azurophiles caractéristiques, sont interprétées comme cellules réticulées non pas comme jeunes cellules myéloïdes. Les résultats cytochimiques diffèrent selon l'espèce des granulations pathologiques. Alors que les résultats de l'analyse qualitative des granulations géantes des cellules myéloïdes sont à peu près normaux, la composition quantitative diffère. Des différences existent entre les inclusions des cellules réticulées et des lymphocytes d'une part et la composition chimique des granulations normales de ces cellules d'autre part. La rétention de ribonucléoprotéines, de polysaccharides et de phospholipoïdes sont les différences les plus importantes.

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Progress in Allergy Vol. 8, Hgb. von *P. Kallós* und *B. H. Waksman*. Karger Verlag, Basel/New York (1964), X + 261 p., 35 Fig. 32 Tab. Preis sF./DM 48.—.

Der 8. Band der Buchreihe «Fortschritte der Allergielehre» umfaßt wiederum ausgewählte Abschnitte des Forschungsgebietes, Experimentelles und Klinisches, in erschöpfender und aktueller Darstellung. Nach einer kurzen Einleitung des Herausgebers P. Kallós wird von P. H. Maurer (USA) über die grundlegenden Untersuchungen zur Aufklärung der Antigenizität mittels synthetischen Polymeren von Aminosäuren berichtet. Nachdem mit synthetischen Polypeptiden Einzelfragen der Antigenstruktur und der genetischen Grundlagen angegangen wurden, weist der Autor – gleichsam als programmatischer Ausblick – auf die Schwierigkeiten hin, die bei den Untersuchungen der natürlich auftretenden Polypeptiden zu erwarten sind. F. L. Adler (USA) orientiert über das umfangreiche Verfahren der Antigenhemmung. Eine große Übersicht wird von E. C. Franklin (USA) über die Immunoglobuline gegeben. Neben Fragen der Struktur und Funktion wird vor allem auf die Methoden der Isolierung, Charakterisierung und Darstellung sowie der entsprechenden Problematik eingegangen. Von E. P. Benditt und D. Lagunoff (USA) wird der Stand der Mastzellforschung umrissen, wobei Funktion und strukturelle Eigenheit gewürdigt werden.

Im mehr klinischen Teil orientieren L. E. Cluff und J. E. Johnson über Fragen des «drug fever», und J. W. Rees (London) diskutiert die Leprominreaktion in bezug auf Epidemiologie und Verlauf der Lepra.

Das Buch ist eine wertvolle Ergänzung der «Progress Series» des Karger Verlages und wird für Allergologen, Immunologen, Mediziner jeder Spezialität sowie für den Therapeuten eine wertvolle Bereicherung der Bibliothek bilden.

U. W. Schnyder, Zürich

M. Amato, D. Catalano: **Istiocitopatie Iperdisplastiche Granulomatose dell'Infanzia.** Minerva medica 1962. 135 p., 91 fig., L. 5000.

Auf dem Gebiete der Retikuloendotheliosen, die von den Autoren Mauro Amato, einem bekannten pädiatrischen Hämatologen, und Dino Catalano, Röntgenologe, als «Istiocitopatie Iperdisplastiche Granulomatose dell'Infanzia» bezeichnet werden, herrschen heute noch große Diskussionen, und man ist sich nicht einmal darüber einig, ob es sich um verschiedene klinische Erscheinungsformen einer einzigen Krankheit handelt oder um verschiedene voneinander scharf zu trennende Krankheitsbilder. Deswegen ist es sehr zu begrüßen, daß die Autoren eine sehr sorgfältige und hauptsächlich histologisch gut belegte kleine Monographie veröffentlicht haben, welche sowohl ihre große Erfahrung vermittelt, als auch versucht, etwas mehr Klarheit in dieses Gebiet zu schaffen. Von vornherein möchten wir sagen, daß dieser Versuch gelungen ist. Zu den bekannten Formen, wie die «Abt-Letterer-Siwe-Krankheit», das «Eosinophile Granulom» und die «Hand-Schüller-Christiansche Krankheit» wird zum erstenmal die von Amato 1956 beschriebene «maligne kongenitale Histiocytopathie» des Neugeborenen eingereiht. Es handelt sich um eine neue Entität, die vom histopathologischen Standpunkt aus zwar der «Abt-Letterer-Siwe-Krankheit» ähnlich ist, im Gegensatz zu dieser Form hingegen bereits bei der Geburt zu schwersten Mesenchymalveränderungen und klinisch-radiologisch nachweisbaren Erscheinungen führt. Die verschiedenen Krankheitsbilder werden systematisch vom klinischen, röntgenologischen und vom histohämatologischen Standpunkt aus betrachtet. Es werden auch die neueren therapeutischen Gesichtspunkte vermittelt. Es handelt sich um eine sehr interessante und kritische Monographie, die außerdem noch typographisch sehr schön und klar ist. Wir möchten den Autoren dazu gratulieren.

E. Rossi, Bern

G. Mathé et *G. Seman*. Aspects Histologiques et Cytologiques des Leucémies et Hématosarcomes. Nomenclature classification, diagnostic, atlas. Librairie Maloine S.A., Paris 1963. 212 p., 38 fig 63 clp., Price F 160.—

In most of the textbooks of hematology the histology of the leukemias and allied disorders is somewhat neglected. In this monograph, *Mathé* and *Seman* have filled this gap admirably

First the normal haematopoetic cells and tissues including lymphnode biopsies and slight modified classification on nomenclature of the leukemias and haematosarcomatoses are presented: the special section discusses in single chapters the histologic and cytologic details of the acute and chronic leukemias (here are also included the polyglobulia Vaquez and the idiopathic-myeloid splenomegaly) the rare forms of leukemia, the reticulo-histiocyto- and histioblastosarcomes, the lymphosarcomes, the myeloma, the macroglobulinemic WALDENSTRÖM, Hodgkin disease, Brill-Symmers, Letterer-Siwe-Hand-Schueller-Christian-disease (Mycosis fungoides) and the eosinophilic granuloma.

The color plates are of the highest quality they are treat to look at! Apart from their great didactic value they clarify many details by juxtaposing peripheral blood, bone marrow and lymphnode histology A carefully selected bibliography brings references of the world literature on leukemias and allied conditions.

This book is very valuable contribution. Certainly it will be widely studied and used it deserves it.

G. ROSENOW New York

Proceedings of the Ninth Congress of the European Society of Hematology Lisbon 1963 S. Karger AG Basel/New York 1964. Part I XX + 118 S., 48 Abb. 8 Tab., Preis F /DM 20.– – Part II/Teil 1, Part II/ Teil 2 XXIV + 1640 S., 437 Abb., 267 Abb. Preis sF / DM 180.–

Die Kongreßberichte sind dank dem direkten Kopieverfahren der vorgetragenen Referate rasch zur Publikation gelangt. Der I. Band enthält die Hauptreferate; der II. Band, in zwei Teilen, die Kurzreferate. Die Vorträge sind nach folgenden Themen eingereiht Ultrastruktur und Cytochemie, Sideroachrestische Anämien, Anämien bei Ernährungsstörungen, parasitäre Anämien, Anomalien des Hämoglobin-Moleküls, Enzyme der Hämoglobin-Synthese, Erythroenzymopathien, Polyglobulien, Hämoglobin-Stoffwechsel, Leukämien und deren Frühstadien, Immunohämatologie, congenitale und erworbene Thrombopathien, Fibrin-Polymerisation und deren Störungen, Fibrinolyse, Radioisotope in der Hämatologie, plasmatische Veränderungen bei Hämopathien und pädiatrische Hämatologie. Während die Hauptreferate eher Übersichten über besondere Gebiete darstellen, enthalten die Kurzreferate das Neueste, was auf den verschiedensten Gebieten der Hämatologie geleistet wird. Die Darstellung ist ausgezeichnet, der Index erlaubt eine rasche Orientierung über das Vorgetragene.

P. FRICK, Zürich

Acta haemat. 32: 129–145 (1964)

Aus der Universitäts-Kinderklinik Tübingen (Direktor: Prof. Dr. K. Betke)

Über die Atemgastransportfunktion des Blutes und die Erythropoese junger Kaninchen*

Von Klaus Riegel und Gerd Ruhrmann

Beim Menschen, bei der Ziege und beim Schaf ändern sich, teilweise in beträchtlichem Umfang, nach der Geburt fast alle morphologischen und funktionellen Größen des Blutes in strenger zeitlicher Folge (22). So kommt es – offensichtlich für jede Species in spezifischem Ausmaß – zu einer lediglich quantitativ und zeitlich unterschiedlichen Abnahme von O_2-Affinität, fetalem Hämoglobinanteil, O_2-Kapazität bzw. Hämoglobinkonzentration, Zahl, Volumen, Oberfläche und Dicke der Erythrocyten und nachfolgend zu einer mehr oder weniger deutlichen Zunahme dieser Größen zu Erwachsenenwerten hin. Auch bei Ratten wurde postnatal trotz maximaler Erythropoese eine Anämie festgestellt (6, 8). Die Anämie wurde auf das relativ größere Körperwachstum bezogen (8). Durch Hypoxie ist erst etwa vom 50. Tag an eine Hämoglobin- und Zellvermehrung zu erreichen (9). Die hinsichtlich der zeitlichen Abfolge zu beobachtenden Differenzen zwischen den Species werden gering, wenn man die Änderungen auf einen einheitlichen Entwicklungsmaßstab bezieht (22). Da die Umweltbedingungen und damit die Anforderungen an den Stoffwechsel für die jungen Individuen der untersuchten Species beträchtlich differieren, erschien es angezeigt, die Abläufe unter Einbeziehung von Knochenmarksanalysen bei einer weiteren Art von primären Nesthockern zu untersuchen. Kaninchen wurden ausgewählt, da über die Gastransportfunktion des Blutes und erythrocytenmorphologische Daten ausgewachsener Tiere reichlich Angaben in der Literatur vorliegen. Es ergab sich, daß – soweit untersucht – im Blut des jungen Kaninchens dieselben Änderungen ablaufen wie bei den wachsenden Individuen der anderen Species.

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* Mit Unterstützung durch die Deutsche Forschungsgemeinschaft.

Meßdaten und berechnete Größen aus dem Blut von Kaninchen verschiedener Altersstufen mit den zugehörigen Gruppenmittelwerten (X̄) T 40 = chemisch gebundene CO_2-Menge des Blutes bei 37 °C, 40 Torr CO_2-Druck und O_2-Vollsättigung. T 50 = O_2-Druck des Blutes bei 37 °C, Serum-pH 7 4 und 50%iger O_2-Sättigung. Zum Vergleich die entsprechenden Daten ausgewachsener Kaninchen nach Literaturangaben.

Gruppe	Alter (Tage)	Gewicht (g)	O_2-Kapazität (Vol %)	Hämoglobin-konzentration (g%)	Erythrozyten-zahl (Mill/mm³)	Hämatokrit (%)	Erythrozyten-[illegible]	Erythrozyten-[illegible]	Erythrozyten-[illegible]	Erythrozyten-[illegible]	Erythrozyten-[illegible]	[illegible]	T 40 (Vol %)	[illegible] (Vol %)	T 50 (Torr)
1	1	68/78	20,38	15,21	4 15	50,5	121,8	60 4	8,77	2.02	176,4	43,8	58,2	56,4	20,4
	1	58/68	17 74	13,24	3,60	43,1	119,7	50,5	8,03	2,38	146,1		52,4	58,5	23,0
	1	60/69	19,85	14,81	4,48	48,0	107 1	55,8	8,43	1,92	162,4		48,6	56,7	20,9
	1	66/68	17,53	13 09	4,51	41,2	91,3	52,3	8,16	1 75	149,3		52,3	59,2	21,0
X̄	1	67	18,88	14 10	4 18	45,7	110,0	54 7	8,34	2,02	158,5	43,8	50,4	57 7	21,3
2	3	57/64	16,91	12,61	4 46	40,2	90,2	53,6	8,42	1 74	158,0	24,3	47 6	53,5	21 7
	3	59/74	13,67	10,20	2,84	33,7	118,7	49,7	7,96	2,39	159 1		64,3	70,5	22,7
	3	94	17 16	12,80	4 17	42,0	100,8	52,8	8,20	1,91	154 7		56,2	64 1	23,3
	5	155	12,78	9,53	3,62	36,0	100,5	53,2	8,23	1,89	155,2		48,9	53,0	23,6
	5	92	15,95	11,90	4,08	42,7	104 6	56,6	8,49	1,85	162,5		59,0	66,5	20,2
X̄	4	85	15,30	11 41	3,83	38,9	103,1	53,6	8,26	1,95	158,0	24,3	55,2	61,5	22,3
3	6	120	15,35	11 43	3,74	38,9	106,7	54 6	8,34	1,96	160 4		53,8	59,5	23,3
	8	211	12,24	9,13	3,34	30,8	92,3	50 6	8,03	1,83	147,2	16,7	35,6	60,0	23,0
	9	117	13,17	9,83	3,29	34,6	105,1	52,6	8,18	2,00	156,6		49,7	53,6	26,7
	9	123	15,25	11,38	4,30	43 0	100,0	53,9	8,28	1,85	158,0	23,0	52,6	58,4	22,5
	10	170	15,26	11,39	3,84	38,8	101,0	55,9	8,44	1,81	159,6		58,0	64,3	25,6
X̄	8	148	14,26	10,64	3,70	37 4	101,0	53,5	8,26	1,89	156 0	20,8	53,9	59,2	24,2
4	13	164	14,90	11 13	4,36	34,5	79 1	48,3	7,86	1,63	137,3	14,0	52,7	58,1	24,0
	13	200	15,97	11,91	4,60	42,6	92,8	49,5	7,94	1,87	145,6		54 4	60,4	23,2
	13	220	15,09	11,25	4 41	43,7	99 1	51 7	8,11	1,92	152,2		50,4	55,7	23,0
	13	180	11 64	8,69				53,2	8,23				54 7	58,5	27,3
	15	240	14 78	11,01	4 46	36,7	82,3	47,0	7 7[illegible]	1 7[illegible]	[illegible]		[illegible]	[illegible]	[illegible]

5	27	303	15,10	12,67	4 79	42,0	87 7	48,7	7,88	1,80	142,0	10,1	36,0	48,3	29 4	
	22	328	16,98	12,25	4,51	37,5	87 0	44,8	7,36	1,94	135,7	9,4	43,2	55,0	31,6	
	25	376	16,40	9,54	4 41	29,6	67 1	44,3	7,50	1,52	124,2		48,9	52,3	31,3	
	25	243	12,79	11 49	5,18	36,6	70,8	41 6	7,27	1 70	122,0		48,0	49,5	31,5	
	35	306	15,40										44,8			
x̄	27	362	15,58	11 49	4,67	36,4	78,1	44,8	7,56	1 74	130,1	9,7	44 4	51,0	30,9	
6	44	677	16,18	12,07	5,72	38,5	67,3	43,2	7,39	1 49	123,9		38,2	42,4	30,7	
	46	1178	15,57	11,62	5 45	39,4	72,3	42,7	7,57	1,09	121,5		46,5	41,2	29,4	
	46	870	16,02	11,95	5,51	38,9	70,6	43,0	7,57	1,57	127,3	8,0	32,0	34,9	30 7	
	52	1050	17,95	13,40	6,03	39,0	64 7	40,6	7 19	1,59	117,2		35,6	41 1	40,4	
x̄	47	944	16,43	12,26	5,68	39,0	68,7	43,4	7 43	1,58	123,7	8,0	38,1	42,4	30,3	
7	62	1350	16,61	12 40	4,84	30,5	73,4	×40,3	7 16	1,87	122,7		40,8	49,2	32,5	
	65	1100	15,.3	11,59	5,88	33,2	58,3	41 1	7,24	1,98	113,4		49,2	42,8	32,2	
	62	1030	17,55	13,10	5,24	38,9	74,3	38,7	7 03	1,92	119,8		44,5	49 7	30,9	
	60	1210	1 77	11 77	4,08	36,1	77 1	44,8	7,55	1 72	130,4	7 0	44,9	54 0	32 4	
	64	1008	15,23	11,96	5,52	36,0	63,2	39,3	7,08	1 66	115 5		39 1	43,9	31,3	
x̄	63	1260	16,14	12,04	5,23	36,2	69 7	40,9	7 15	1 71	120,4	7,0	43,7	47,9	31,9	
Ausgewachsene Kaninchen																
Bergstra (4)		3300	14,55	10,86	5,38	32,6	63,5		6,80	1,80	111 0	3,3	47 0	51,0	30,3	7
Bartels und Harms (2)			13,93	10,40											30,7	6
Rittelard, Kunz (17 15)		3300	16,83	12,56	5,44	36,6	67,3	39 1	7,06	1 73	116,8	2,2	36,2	42,9	32,3	16
x̄			15,68	11 70	5,41	35,4	60,8		6,90	1 75	114,8	2,6	39,5	46,3	31,5	

Methodik

Versuchsgang. Wir untersuchten 8 Würfe gesunder Inzuchtkaninchen mit insgesamt 45 Tieren. Die einzelnen Tiere wurden nur einmal untersucht, um durch die Entnahme von jeweils 5–8 ml Blut die weiteren Meßwerte nicht zu beeinflussen. Da die Würfe unterschiedlich groß waren, konnte nicht für jede Altersgruppe ein Tier jeden Wurfes herangezogen werden. Die Gruppeneinteilung – 1.–4., 8., 13. Tag, zwischen 22. und 35.–44. und 52. sowie 62. und 65. Tag – erfolgte willkürlich. Den Tieren wurde ohne vorherige Karenz vormittags gegen 9 Uhr das Blut entnommen. Bis zum 5. Tag durch Dekapitieren, bis zum 40. Tag durch Herzpunktion, dann aus einer Ohrvene. In den ersten 3 Tagen waren für eine Untersuchung zwei Tiere nötig. Die Gerinnung wurde durch Zugabe von pulverisiertem Heparin, die Glykolyse in den Proben für die Gasanalysen durch Zugabe von Natriumfluorid verhütet. Das Knochenmark wurde durch Eröffnung des Femur bei den jungen, durch senkrechte Punktion der Tibia vom Kniegelenkspalt bei den älteren Kaninchen gewonnen.

Bestimmungen. Die Blutgasanalysen zur Bestimmung der O_2-Affinität, O_2-Kapazität, des bei 37° 40 Torr CO_2-Druck und O_2-Vollsättigung im Blut (T 40) und im Serum (Standardbikarbonat) chemisch gebundenen Kohlendioxyds erfolgten nach den Angaben von Bartels und Harms (2). Aus der O_2-Kapazität wurde mit der Hüfnerschen Zahl die Hämoglobinkonzentration berechnet (12). Die Erythrozytenzahl in der Neubauerkammer und der Hämatokrit mit der Mikrohämatokritzentrifuge von Hawksley wurden vierfach bestimmt. Der Durchmesser der roten Zellen wurde planimetrisch ermittelt (10). Das weitere Vorgehen ist früher beschrieben worden (22).

Die Knochenmarkspunktate wurden nach vorsichtigem Ausstreichen, anschließender Lufttrocknung und Pappenheim-Färbung differenziert. Die Ausstriche von 25 Tieren aus 6 Würfen wurden ausgewertet. Wir differenzierten mindestens 500–600 Zellen pro Tier, insgesamt 16800 Zellen. Die Relation zwischen Erythropoese und Myelopoese wurde errechnet und die Mittelwerte für den 1., 3., 6., 9., 13., 45. und 90. Tag bestimmt.

Bei der *Berechnung* der Gesamt-Erythrozyten- und Hämoglobinmenge wurde, entsprechend den Literaturangaben für erwachsene Tiere (1), ein Blutvolumen von 7% des Körpergewichtes angenommen. Nicht berücksichtigt wurde ein eventuell höheres Volumen des Neugeborenen, da unseres Wissens keine Unterlagen über den Verlauf dieser Größe beim Kaninchen vorliegen. Die Menge der roten Vorstufen im Mark wurde über die Höhe der Retikulozytenzahl berechnet, da nach den bisher vorliegenden Erythropoesemodellen die Retikulozytenzahl der Zahl der orthochromatischen Erythroblasten entsprechen sollte.

Die statistischen Berechnungen erfolgten nach den Angaben der Documenta Geigy, 6. Auflage 1960.

Ergebnisse

Die Resultate von den einzelnen Tieren sind in Tabelle I zusammengefaßt, die Gruppenmittelwerte in den Abb. 1 bis 3 graphisch wiedergegeben.

O_2-Kapazität (Hämoglobinkonzentration), *Erythrozytenzahl je mm³* und *Hämatokrit* werden unmittelbar nach der Geburt reduziert zu einem Tiefstwert in der 2. Lebenswoche. Die O_2-Kapazität von etwa 19 auf 14,2 Vol% (Hämoglobin von 14 auf 10,5 g%), die

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Dankenswerterweise überlassen von der Deutschen Hoffmann-La Roche AG, Grenzach/B.

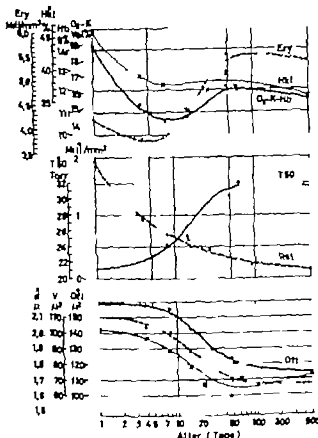


Abb. 1 Verläufe der wichtigsten Meßergebnisse im Blut des Kaninchens in der ersten Lebenszeit (Abszisse: Alter in Tagen in logarithmischer Darstellung) O_2-Kapazität (O_2-K Vol%) bzw. Hämoglobinkonzentration (Hb g%) Erythrozytenzahl (Ery Mill/mm³) und Hämatokrit (Hkt %) nehmen nach der Geburt in der 1. Lebenswoche ab, bis zum 50. Tag zu und nähern sich dann dem Erwachsenenmittel. Die Retikulozytenzahl (Ret Mill/mm³) nimmt kontinuierlich ab. Der O_2-Halbsättigungsdruck (T 50 Torr) nimmt nach der Geburt zu und erreicht um den 50. Tag das Erwachsenenmittel. Die morphologischen Abmessungen mittlere Oberfläche der Erythrozyten (Oß μ²), mittleres Einzelvolumen (V μ³) und mittlere Dicke (d μ) nehmen nach der Geburt ab und erreichen den jeweiligen Erwachsenenwert ebenfalls um den 50. Tag, nur die Dicke liegt um diese Zeit noch etwas darunter

Erythrozytenzahl von 4,2 auf 5,7 Mill., der Hämatokrit von 46 auf 37% (Abb. 1). Danach erfolgt, bei O_2-Kapazität und Erythrozytenzahl eindeutig, eine Vermehrung, die im Alter von rund 50 Tagen ein Maximum erreicht, und schließlich eine langsame Annäherung an die Erwachsenenwerte. Die Retikulozytenzahl nimmt nach der Geburt stetig ab. Die Hämoglobinkonzentration in den Erythro-

Tabelle II

Daten der O_2-Bindungskurven des Blutes junger Kaninchen verschiedener Altersstufen, angegeben für 37 °C und Serum-pH 7 4 mit den Gruppenmittelwerten ($\bar{X}$).

		T[illegible]	T[illegible]	T[illegible]	T[illegible]	T_{70}	T[illegible]	T[illegible]	T[illegible]
Gruppe 1				20,4	23,2	26,6	30,5	33,1	38,0
1 Tag				23,0	26,1	29,9	35,2	39,3	44,8
				20,9	23,8	27 4	32,2	33,8	40,7
				21,0	23,9	27 4	32,3	35,9	41,3
	$\bar{X}$			21,3	24,2	27,8	32,6	36,0	41,2
Gruppe 2				21,7	24,5	28,1	33,0	37 1	43,6
3–5 Tage				22,7	25,8	29,6	33,0	38,9	44 4
				23,3	26,4	30,3	35,7	39,7	43,3
				23,6	26,9	30,8	36,4	40,5	46,8
				20,2	23,0	26,4	31 1	34 4	39,3
	$\bar{X}$			22,3	25,3	29,0	34,2	38,1	43,9
Gruppe 3				22,5	25,7	29,5	34,7	38,5	43,9
6–10 Tage				23,0	26,2	30,1	35,5	—	—
				23,3	26,4	30,3	35,7	39,7	43,3
				26,7	30,3	34,8	40,8	45,2	51,3
				25,6	29 1	33,3	39,5	44,0	51,3
	$\bar{X}$			24,2	27,5	31,6	37,2	41,8	47,9
Gruppe 4				24,0	27,3	31 4	36,7	40,2	43,6
11–20 Tage				25,2	28,7	33,0	38,9	43,2	48,6
				23,0	26,5	30,5	35,6	40,7	47,2
				27,3	30,9	36,1	42,4	47 4	54,2
				26,4	30,0	34 4	40,1	43,7	49,5
	$\bar{X}$			25,2	28,7	33,0	38,7	43,0	48,6
Gruppe 5			25,6	29,4	33,7	38,7	45,8	50,9	
21–40 Tage			27,5	31,6	36,0	41,3			
			27,2	31,3	35,9	42,0	50,1		
			27 4	31,5	35,9	41,3			
	$\bar{X}$		26,9	31,0	35 4	40,8	48,0		
Gruppe 6			26,7	30,7	34,9				
41–60 Tage			25,5	29,4	33,4	38,4	44,9	49,8	
		22,7	26,7	30,7	34,9	40,0			
			26,4	30 4					—
	$\bar{X}$		26,3	30,3	34 4	39,2			
Gruppe 7		23,1	24,3	31,5	33,7	40,6	47,8		
>60 Tage		24 0	28,3	32,4	36,6	42,2	50,1		
		22,8	26,9	30,9	34,9	39,8	46,4		
		23,8	28,0	32,5	36,9	42,9	50,3		
		23,8	28,0	32,2	36,4	41,6	48,9		
	$\bar{X}$	23,5	27 1	31,9	36,1	41,5	48,7		

den. Da dies hier nicht zur Diskussion steht, werden die Ergebnisse in anderem Zusammenhang mitgeteilt.

Die *O_2-Bindungskurven des Blutes* werden, unmittelbar nach der Geburt beginnend, nach rechts verschoben. Ausgedrückt in O_2-Druckwerten bei 50 %iger O_2-Sättigung des Hämoglobins (T 50) ergibt sich eine Zunahme von rund 21 auf 32 Torr nach 2 Monaten (Abb. 1) Der T 50-Wert ausgewachsener Tiere beträgt bei vergleichbaren Bestimmungen 31 Torr (2 4 15) Nach dem 50 Tag wird dieser Wert nur geringfügig überschritten. Tabelle II enthält die Werte der O_2-Bindungskurven der einzelnen Tiere und der Gruppenmittel sowie die entsprechenden Daten ausgewachsener Tiere, jeweils angegeben für 37 C. Zur Umrechnung auf die reale Körpertemperatur des Kaninchens sind die Faktoren von SCHLOSSER UND BARTELS* zu verwenden. Für 39 C ergibt sich eine Zunahme des T 50-Wertes von 3 6 Torr

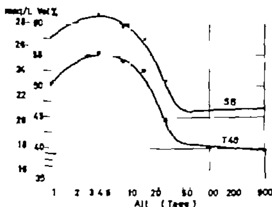


Abb. 4. Die CO_2-Transportgrößen des Blutes (T 40 und Standardbikarbonat Vol% bzw. mVal/L) im Verlauf der ersten Lebenszeit des Kaninchens. Die CO_2-Parameter nehmen bis zum 4 Tag zu, nachfolgend bis zum 50. Tag ab, jetzt das Erwachsenenmittel erreichend.

Die *CO_2-Parameter* [chemisch in Blut *(T 40)* und Serum *(Standardbikarbonat)* bei 37 C, 40 Torr CO_2-Druck und O_2-Vollsättigung gebundene CO_2-Menge] ändern sich nach der Geburt beträchtlich (Abb. 4) Der T-40-Wert nimmt in den ersten 5 Tagen von etwa 50 auf 55 Vol% zu und nachfolgend bis zum 50 Tag auf ein Minimum von 38 Vol % ab, um sodann zum Mittelwert ausge

* Unveröffentlicht.

wachsener Tiere (46 Vol %) langsam anzuwachsen. Der Standardbikarbonatgehalt zeigt denselben Verlauf die Differenz zum T-40-Wert ist lediglich von der Hämoglobinkonzentration abhängig

Tabelle III

Koeffizienten korrelierter Größenänderungen im Blut junger Kaninchen. Es gilt ein Zufallshöchstwert von 0,51

X	Y		
	O_2-Affinität T 50	CO_2-Gehalt T 40	Standard-bikarbonat
O_2 Kapazität	+0,39	—0,52	—0,71
Erythrozytendurchvolumen	—0,83		
Erythrozytenoberfläche	—0,89		
Erythrozytendicke	—0,52		

Über etwaige Korrelationen zwischen den einzelnen Größen orientiert Tabelle III Wie in vorausgehenden Untersuchungen konnten bei der Gegenüberstellung von T 50 und Erythrozytenoberfläche (r = —0 89) bzw Zellvolumen (r = —0 83) hohe Korrelationskoeffizienten errechnet werden, während T 50/Dicke nur knapp zu sichern ist (r = —0,52) Zwischen der Höhe der O_2-Kapazität und der O_2-Affinität läßt sich kein Zusammenhang ableiten auch nicht zwischen O_2-Kapazität und dem T-40-Wert – der höhere Korrelationskoeffizient (r = 0 71) der Relation O_2 Kapazität zu Standardbikarbonat beweist in diesem Zusammenhang nichts.

Diskussion

Trotz überwiegender und noch zunehmender Erythropoese resultiert beim jungen Kaninchen in den ersten Tagen nach der Geburt eine Reduktion von O_2-Kapazität bzw Hämoglobinkonzentration, Zellzahl und Zellmasse je Volumeneinheit Blut – durchaus vergleichbar der Trimenonreduktion beim Menschen oder dem Verhalten dieser Werte bei Schaf und Ziegenlamm. Der Gesamtbestand an peripheren Zellen und Hämoglobin folgt indessen ziemlich genau der Gewichtszunahme (Abb. 5) Die postnatale relative Reduktion bedeutet beim Gesamthämoglobin ein Gleichbleiben des Wertes in den ersten 5 Tagen. Umgerechnet auf die Gewichtseinheit kg findet sich ein durchaus dem Menschen vergleichbarer Verlauf (14) Ausgehend von 10 g/kg bei der Geburt kommt es zu einem Tiefwert von 7,5 g am 8. Tag zu einem Anstieg

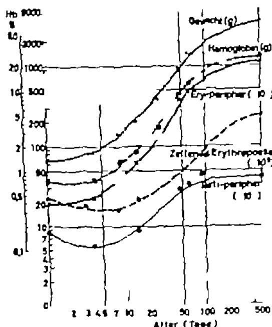


Abb. 5. Das Verhalten des Gewichtes, der Hämoglobinmenge und der Erythrocytenzahl im peripheren Blut in Relation zur gesamten Zellzahl der Erythropoese im Knochenmark beim Kaninchen in der ersten Lebenszeit. Abszisse und Ordinate in logarithmischem Maßstab. Erklärung im Text.

auf 8,6 g in der 7. Woche und wieder zu einem Abfall auf etwa 8 g Hämoglobin/kg bei ausgewachsenen Tieren. Der errechnete absolute Zellbestand der roten Vorstufen im Mark weist demgegenüber eine deutliche Depression bis zum 8. Tag auf, obwohl das relative Verhältnis von Erythropoese zur Granulopoese gerade zu diesem Zeitpunkt maximal überwiegt. Die Untersuchungen bestätigen also eindeutig die Ansicht von GARCIA (8). Die Gewichtszunahme nach der Geburt ist prozentual stärker als die Neubildung von Erythrozyten, der Gesamtbestand an peripheren Zellen und zirkulierendem Hämoglobin unterschreitet aber zu keiner Zeit den Wert bei der Geburt. Die bei der Geburt die Granulopoese schon stark überwiegende Erythropoese muß in den ersten Tagen noch erheblich zunehmen, damit die periphere Zellmasse erhalten bleibt.

In anderer zeitlicher Abfolge vollzieht sich eine Umstellung der Zellform. Die in allen Dimensionen großen fetalen Zellen werden im Verlauf von 7 Wochen durch eine postfetale Population ab-

gelöst, die Zellen von längerer Lebensdauer benutzt (60 bis 65 Tage, 5 27) Dieser Ablauf ist streng mit einer Änderung der Gastransportfunktion der roten Zellen korreliert.

Die O_2 Transportgröße des Blutes wird von der Höhe der Hämoglobinkonzentration (O_2 Kapazität) und den O_2 Bindungseigenschaften des roten Farbstoffes (O_2-Affinität) bestimmt. Der Hämatokrit ist unmittelbar von der Hämoglobinkonzentration abhängig da die Packung des Hämoglobins in der Zelle nur unerheblich schwanken darf, wenn eine optimale Funktion gewährleistet sein soll. Die Zahl der Erythrozyten ist hinsichtlich ihrer Gastransportfunktion relativ unwichtig weshalb die Zellneubildung sicher nicht über die periphere Erythrozytenzahl reguliert wird, wie von Ruhenstroth Bauer (23) angenommen wird. Die Form der Erythrozyten ist indessen nicht gleichgültig Vor allem der Weg, den der Sauerstoff bei der Aufsättigung der Hämoglobinmoleküle im Zellinnern zurücklegen muß d h die Dicke, kann als limitierende Größe angesehen werden (22)

Eine Aussage über die Höhe der O_2 Versorgung der Gewebe erlaubt die Höhe der O_2-Ausschöpfbarkeit des Blutes. Diese ergibt sich als Resultierende aus O_2 Affinität + O_2-Kapazität im O_2-Druckbereich zwischen arteriellem und venösem Blut. Da wir den venösen O_2-Druck aus Vergleichsgründen mit 30 Torr konstant annehmen, ist die O_2-Ausschöpfbarkeit eine fiktive Größe.

Den venösen O_2-Druck bei 30 Torr als konstant anzunehmen hat einige Berechtigung So hatten Versuche an jungen Ziegen und Schafen ergeben, daß der O_2-Druck in der A. pulmonalis einmal nahe bei 30 Torr während der ganzen Beobachtungszeit lag und recht wenig davon abwich (3) Dem entspricht die Bestimmung im venösen Mischblut bei zwei 13 Tage alten Kaninchen. Zum andern kann als gesichert gelten, daß niedrigere O_2-Drucke sofort mit einer Steigerung des Herzminutenvolumens beantwortet werden (11) Schließlich ist z.B. für das Gehirn die Einhaltung eines Gewebs-O_2-Grenzdruckes von wesentlicher funktioneller Bedeutung (26) Das schließt nicht aus, daß in anderen Organen, wie Herz oder arbeitendem Skelettmuskel, der O_2-Grenzdruck beträchtlich niedriger liegen kann.

Die O_2-Ausschöpfbarkeit muß mindestens so groß sein wie die reale O_2-Differenz zwischen arteriellem und gemischtvenösem Blut ($AVDO_2$) wenn das Herzminutenvolumen nicht gesteigert werden soll. Tatsächlich ist sie z.B. beim Menschen immer größer mit Ausnahme des kurzen Abschnittes zwischen 2. und 4 Lebensmonat, wo sich die beiden Größen entsprechen. Es erfolgt nach der Geburt eine Reduktion, die auf die Hämoglobinabnahme zu beziehen ist, danach eine Zunahme auf den Erwachsenenwert hin. Beim Kanin-

chen ergibt sich dasselbe Verhalten (Abb 3) Eine Abnahme von 4,2 auf 3 7 Vol% am 4 Tag dann allerdings eine Zunahme auf 7,5 Vol% Ende des 2 Monats über den Erwachsenenwert (6 Vol%) hinaus. Auch bei Ziege und Schaf kommt es im Gegensatz zum Menschen im 2./3 Trimenon zu einer beträchtlich höheren O_2 Ausschöpfbarkeit als beim ausgewachsenen Tier (22)

Nach dem oben Ausgeführten müßte die $AVDO_2$ des Kaninchens etwa 4 Vol% betragen Nachdem darüber keine Literaturangaben vorliegen, haben wir einige Herzpunktionen für die Analyse aktueller Blutgase verwendet (Tabelle IV) Exakte Angaben über die Verhältnisse in arteriellem und gemischtvenösem Blut waren bei diesem Vorgehen nicht zu erwarten Indessen wird man den Messungen entnehmen können, daß die $AVDO_2$ des Kaninchens nahe 4 Vol% liegt Eine darüber hinausgehende O_2 Ausschöpfung des Blutes muß zu einer Erniedrigung des venösen O_2 Druckes führen. Wir sehen die von Geburt an gesteigerte und bis zum 9 Tag noch zunehmende Erythropoese in direktem Zusammenhang mit der O_2-Ausschöpfbarkeit des Blutes, die ein guter Indikator für die angespannte O_2-Versorgungs- und Zellnachschublage sein dürfte.

Auffällig mag in Tabelle IV der niedrige arterielle O_2-Druck erscheinen. Er liegt jedoch in demselben Druckbereich, den wir (3, 21) bei jungen Zicklein und Lämmern in vergleichbarem Alter sowie beim jungen menschlichen Säugling ermitteln konnten. Der arterielle O_2-Druck ist in den ersten Lebenstagen beim Kaninchen vermindert, der venöse Druck liegt mit Wahrscheinlichkeit niedrig.

Die Abhängigkeit der Neubildung der roten Zellen von dem Ausmaß der O_2 Versorgung des Organismus ist durch zahlreiche Beobachtungen gesichert. Die ausführlichsten Untersuchungen

Tabelle IV

Untersuchungsergebnisse aktueller Blutgasparameter junger Kaninchen 8 bis 27 Tage alt. Blutproben gewonnen durch Blindpunktion. Die Resultate sprechen dafür daß drei Proben aus dem linken und zwei aus dem rechten Herzen stammen.

Alter (Tage)		O_2-Kapazität (Vol %)	O_2-Gehalt (Vol %)	O_2-Sättigung (%)	O_2-Druck (Torr)	pH	CO_2-Druck (Torr)
arterielle	8	12,84	12,39	96,5	56,2	7 412	43,2
	9	15,25	15,09	98,9	57 7	7 400	47,6
	27	15,12	14 46	95 6	73,0	7 407	51 7
venöse	13	13 45	9,06	67 4	28,6	7.316	60,6
	13	14,90	10,58	71 1	30,9	7.335	48,4

haben Hurtado et al. (13) durchgeführt. Der O_2-Mangel hat eine indirekte Wirkung auf das Knochenmark, zwischengeschaltet sind Erythropoetine. Als deren wichtigste Bildungsstätte sind die Nieren anzusehen (19). Der wichtigste Rezeptor für den O_2-Mangel wird indessen in das Zwischenhirn lokalisiert. Als adäquater Reiz ist der O_2-Grenzdruck im Gewebe anzusehen (18), der, wie aus dem bisher Ausgeführten hervorgeht, unmittelbar vom O_2-Verbrauch des Gewebes und von der O_2-Ausschöpfbarkeit des Blutes abhängt.

Bei einem verminderten arteriellen O_2-Druck, sei es bei einem Aufenthalt in großer Höhe oder bei einer Mischblutzyanose, wird die O_2-Kapazität, d. h. die Hämoglobinkonzentration im Blut, in dem Umfang gesteigert, bis bei ausreichendem O_2-Gewebsdruck eine normale O_2-Ausschöpfbarkeit gewährleistet ist. Ist die O_2-Kapazität erniedrigt fixiert, z. B. bei Anämien, kommt es zu einem *Kompensationsversuch von seiten der O_2-Affinität*. Die meisten Anämien können bis zu einem gewissen Grad (etwa 8 g%) als kompensiert gelten (20). Bei ausreichendem O_2-Angebot kann schließlich die O_2-Versorgung der Gewebe ungenügend werden, wenn die Atmungsfermente blockiert sind. Diese Aussagen gelten für alle Organe mit einigermaßen konstantem Stoffwechsel (Niere, Leber).

Die morphologischen Daten des Knochenmarkes bestätigen die große Kapazität der Erythropoese, die vom wachsenden Organismus gefordert und geleistet wird. Es verdient hervorgehoben zu werden, daß bisher nur aus den *relativen* Beziehungen der Zellsysteme beschränkte Schlüsse möglich sind. Eine echte quantitative Betrachtungsweise ist erst gegeben, wenn Daten über den *absoluten* Knochenmarksanteil am Körperwachstum vorliegen. Da für den Säugling entsprechende Untersuchungen aus methodischen Gründen fehlen, wäre wenigstens ihre Kenntnis am wachsenden Tier von großem Interesse. Absolute Knochenmarkswerte wurden bisher nur an erwachsenen Tieren (Meerschweinchen 27, Kaninchen 16) mitgeteilt.

Für die hier vorgelegten *relativen* Werte beim Kaninchen gibt es aus der Literatur lediglich Hinweise bei Sabin et al. (24). Rechnet man die angegebenen Zellzahlen um, dann ergeben sich in der 2. Woche ebenfalls die höchsten Zahlen: 326/100 bei einem allerdings entsprechend niedrigeren Ausgangswert am Tag der Geburt von 149/100, also im Prinzip die gleiche Steigerung, wie wir sie bei unseren Tieren beobachten konnten.

Das junge Kaninchen hat nach diesen im wesentlichen gleich lautenden Aussagen ein Maximum an Erythropoese zu dem Zeitpunkt, an dem es sein Gewicht verdoppelt hat, und vermindert seine Erythropoese mit abnehmender Wachstumsgeschwindigkeit.

Zusammenfassung

Untersuchungen an neugeborenen und wachsenden Kaninchen zeigen, daß die O_2-Kapazität, die Erythrocytenzahl und der Hämatokritwert von Geburt an bis in die zweite Lebenswoche abnehmen. Auch die Retikulocytenzahl nimmt rasch ab. Die morphologischen Daten der Erythrocyten (Volumen, Oberfläche, Dicke und Durchmesser) verringern sich merklich erst von der 2. Lebenswoche bis zu 50 Tagen. Die Erythropoesezellen im Knochenmark, die bei der Geburt die Granulopoese im Verhältnis von 300/100 Zellen überwiegen, steigern sich bis zum 9. Tag noch auf 520/100 und erreichen abfallend erst am 45. Tag ungefähr die Relation des erwachsenen Kaninchens. Die O_2-Bindungskurven des Blutes werden unmittelbar nach der Geburt beträchtlich nach rechts verschoben. Auch die CO_2-Parameter ändern sich, der T-40-Wert nimmt in den ersten 5 Tagen zu und sinkt dann bis zum 50. Tag auf ein Minimum ab. Die gewonnenen Daten werden in Tabellen und Kurven übersichtlich mitgeteilt und mit den bisher bekannten Vergleichswerten wachsender Tiere und der menschlichen Entwicklung diskutiert.

Summary

Investigations in newborn and growing rabbits show that the O_2 capacity erythrocyte count and haematocrit values decrease from birth until the second week of life. The number of reticulocytes also decreases rapidly. Volume, surface area, thickness and diameter of the erythrocytes diminish notably only between the second week and the fiftieth day of life. Erythropoietic cells in the bone marrow, which at birth exceed the granulopoietic cells by 300 100 increase to a ratio of 520 100 by the ninth day falling to about the adult proportion only after 45 days. The curve of O_2 uptake in the blood is shifted markedly to the right immediately after birth. CO_2 levels are also altered: in the first five days, T-40 values increase, to fall to a minimum after 50 days. The data obtained are tabulated and discussed in relation to those already available for growing animals and human development.

Résumé

Des recherches faites chez des lapins nouveaux-nés et en période de croissance montrent, que la capacité en oxygène, le nombre des érythrocytes et la valeur de l'hématocrite diminuent dès la naissance jusqu'à la 2e semaine de vie. Le nombre des réticulocytes décroît aussi rapidement. Le volume, la surface, l'épaisseur et le diamètre ne diminuent notablement qu'entre la 2e semaine et le 50e jour de vie. Les cellules érythropoïétiques de la moelle osseuse, qui à la naissance excèdent les cellules granulopoïétiques de 300 100, augmentent encore leur proportion à 520 100 le 9e jour et tombent seulement vers environ 45 jours aux proportions des lapins adultes. La courbe d'oxygénation du sang est considérablement déviée vers la droite immédiatement après la naissance. Les taux de CO_2 sont aussi modifiés: dans les premiers 5 jours, les valeurs T 40 augmentent, puis tombent à des valeurs minimales après 50 jours. Les résultats sont établis dans des tableaux et comparés aux valeurs connues d'autres animaux et du développement humain.

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Technique of Transfusion. Thirteen days after sub-lethal irradiation of the donor animal its bone marrow was removed and transfused into litter-mate. Twenty four hours previously the litter-mate had itself received either 600 or 720 rads whole body irradiation. The donor was anaesthetised with ether and exsanguinated by incising the inferior vena cava. The blood was centrifuged to provide serum for preparing smears from sample of the donor bone marrow The long bones of the limbs (femur and humerus on both sides) were removed and cleaned of muscle. The ends of each bone were scored with bone-saw and snapped off, to expose bone marrow at each end of the bone. Initially the bone cortex was removed with nibblers and the marrow extracted with an elevator and placed in 2 mls. of Culture Medium 199' at 37 °C. In later experiments the marrow was removed by perfusing the bone cavity with culture medium It was suspended in the medium by rapid agitation. Two samples of the suspension were diluted in WBC counting fluid and the nucleated cells enumerated in haemocytometer Although the fat content of the marrow obtained from the donor animals is higher than normal (29) the fat was retained in the marrow suspension injected into the lethally irradiated animals. The recipient guinea-pig was anaesthetised with ether and the suspension injected into the internal jugular or the external iliac vein exposed through a small skin incision. The incision was closed with thread sutures and the wound sealed with an aerosol collodion spray Invariably the wound healed promptly The interval between removal of bone marrow from the donor animal and its injection into the recipient was usually less than 20 minutes.

Cellular Analysis of the Transfused Bone Marrow A sample of the donor' bone marrow was suspended in small volume of isologous serum. Smears were prepared from this suspension, air-dried and fixed in methyl alcohol. They were stained in an aqueous dilution of MacNeal Tetrachrome at pH 5.5 and mounted in picolyte. A differential count was made of 2,000 nucleated cells, using four smears for the count.

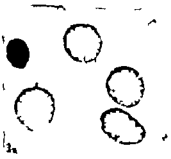
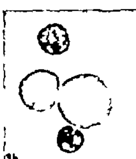
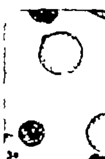
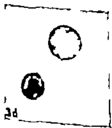
The Number of Bone Marrow Cells Transfused. In guinea-pigs receiving 600 rads whole body irradiation, the mean number of bone marrow cells transfused into each animal was $88.5 \pm 13.7 \times 10^{6}$ and in those given 720 rads the average number of cells transfused was $115.2 \pm 17.7 \times 10^{6}$

Results

Cellular Composition of the Transfused Bone Marrow

Details of the cellular composition and proliferative activity in bone marrow of guinea pigs in the early stages of final haemopoietic recovery following sub-lethal whole body gamma irradiation have been previously published (29 30 31) Bone marrow at this stage of regeneration was used for transfusion in the present experiments and differential counts of samples of the transfused marrow are recorded in table I A representative area illustrating the types of cells in a smear of transfused marrow is shown in fig 1 Its appearance is in marked contrast to that of normal bone marrow (fig 2) The most striking feature of the bone marrow used for transfusion is that it contains only a very small percentage of cells in the erythroid and granulocytic groups, the early forms being particularly scarce. The bulk of the transfused cells (70–80%) are



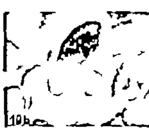








Fig 1 Representative field in smear of guinea-pig bone marrow illustrating the cell-types in the transfused bone marrow MacNeal Tetrachrome, × 200.

Fig 2. Smear preparation of normal guinea-pig bone marrow MacNeal's Tetrachrome × 200.

Fig 3a, b, c, d. Mononuclear cells of transitional morphology in smears of guinea-pig bone marrow used for transfusion. MacNeal Tetrachrome, × 970.

Fig 10 a, b d, f g h. Unusual forms of leucocytes appearing in the peripheral blood of animals within two weeks of bone marrow transfusion MacNeal Tetrachrome × 920.

Table I
Cellular composition of transfused bone marrow (%)

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Granulocytes					
Myeloblast	2.80	4.00	2.40	1.50	3.45
Promyelocyte	—	—	0.05	0.05	0.10
Neutro. Myelocyte	0.05	0.05	—	0.15	0.10
Neutro. Metamyelocyte	0.25	0.10	—	—	—
Neutro. Band Cell	2.25	3.30	0.05	0.10	0.20
Neutro. Polymorph.	3.65	2.05	1.10	0.65	0.80
Neutro. Total	6.20	5.50	1.15	0.90	1.10
Eosino. Myelocyte	0.10	—	0.05	—	—
Eosino. Metamyelocyte	0.10	0.10	—	—	—
Eosino. Band Cell	0.15	0.05	0.05	0.15	0.35
Eosino. Total	0.35	0.15	0.10	0.15	0.35
Baso. Metamyelocyte	—	—	—	0.05	—
Total Granulocytes	9.35	9.65	3.70	2.65	5.00
Erythroid					
Proerythroblast	0.85	0.15	0.65	1.60	1.25
Baso. Erythroblast	1.15	—	0.50	1.75	0.95
Poly. Erythroblast	1.80	—	0.45	1.50	0.70
Ortho. Erythroblast	2.60	0.20	0.15	1.80	1.55
Total Erythroid	6.40	0.35	1.75	6.65	4.45
Lymphoid					
Small Lymphocyte	40.85	46.55	[illegible]4.35	53.15	42.05
Medium Lymphocyte	12.80	13.60	14.60	11.95	17.25
Total	53.65	60.15	68.95	65.10	59.30
Transitional Cell	16.95	16.15	11.[illegible]5	8.60	12.25
Total Lymphoid	70.60	76.30	80.50	73.70	71.55
Plasma Cell	0.35	0.10	0.15	0.30	0.80
Reticulum Cell (including phagocytic forms)	0.15	0.20	0.80	0.50	1.70
Megakaryocyte	—	—	—	—	0.05
Monocyte	—	—	—	—	0.05
Damaged Cell	13.15	13.40	13.10	16.20	16.40

classified in the lymphoid series. These are mononuclear cells of various sizes with a basic morphology. The nucleus is rounded and occupies most of the cell. The cytoplasm is scanty and forms a thin rim at one side of the nucleus or occupies a small nuclear indentation. Most of these cells (76—88 / in the samples counted) resemble

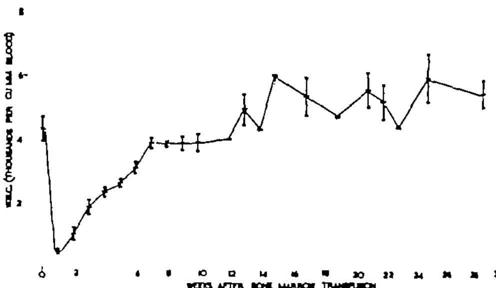


Fig. 4 a, b, c. Changes in the total WBC, neutrophil and lymphocyte levels of peripheral blood in guinea-pigs transfused after 720 rads whole body gamma irradiation.

Note. The sign ┬ on the graphs in figs. 4, 5, 6, 7, 8 and 9 indicates the mean of the observations and the *standard error* of the mean, except in the case of single observations which are indicated thus: —

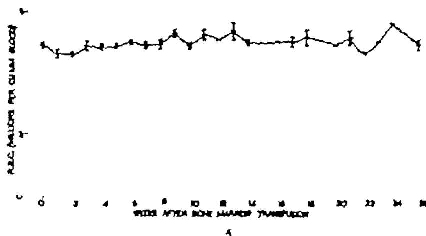
small and medium sized lymphocytes. The remainder have a transitional morphology partly resembling blast cells, and partly lymphocytes. Their characteristics have been fully described and illustrated previously (29 30 31 51) They range in size from the smaller form of lymphocyte to cells approaching the size of a proerythroblast and myeloblast. Examples are illustrated in fig 3 The incidence of damaged cells in smears of the irradiated marrow is higher than in smears of normal marrow and is related to the increased fat content of the irradiated marrow (29) There is evidence that cells of all sizes contribute to the damaged fractions (31)

Animals Transfused after 720 rads Irradiation

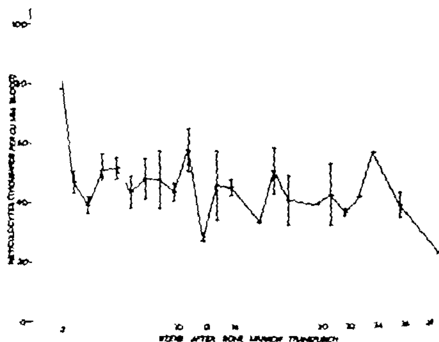
WBC Levels The changes in the total WBC, neutrophil and lymphocyte levels in the peripheral blood of guinea pigs up to 29 weeks after transfusion are shown in figs. 4 a, 4 b and 4 c. A profound leucopoenia is evident 7 days after transfusion and this involves both neutrophils and lymphocytes. However by the 14th day the neutrophil levels are restored to within normal limits and

WEEKS AFTER BONE MARROW TRANSFUSION

WEEKS AFTER BONE MARROW TRANSFUSION



5



5b

Fig. 5 a, b. Changes in the RBC and reticulocyte levels of peripheral blood in guinea-pigs transfused after 720 rads whole body gamma irradiation.

in the subsequent 5 weeks they remain at only slightly sub-normal, and apparently adequate, levels. Thereafter the neutrophil count is predominantly within normal limits although in later weeks there is a tendency in some animals for the count to reach values which are rather high for normal guinea pigs. The restoration of blood lymphocytes is protracted and is in marked contrast to the rapid recovery of neutrophils. From the 1st week after transfusion, their levels rise only very slowly and do not reach the normal range until the 8th week. After the 14th week the lymphocyte levels are rather higher than the normal values for young guinea pigs.

RBC and Reticulocyte Levels The red cell levels remained almost normal after transfusion, there being only a very slight depression during the 1st and 2nd weeks (fig 5a) The absence of anaemia was reflected in the general progress of the transfused animals. They remained very active after transfusion and retained excellent appetites. The blood reticulocytes were depressed below normal levels for the 1st week after transfusion but an appreciable reticulocytosis developed during the 2nd week (fig 5b) Subsequently the reticulocyte count remained about the normal value.

Body Weight. This was a reliable index of progress after transfusion. A very small and statistically insignificant decrease in weight was evident at the end of the 1st week but subsequently body weight increased steadily (fig 6) to eventually reach values comparable with those of normal animals.

Long-term Survival Of a total of 10 transfused animals, 9 have survived to the time of writing The survival times vary between 1 and 2.25 years. One animal died having survived almost 2 years after transfusion. Post-mortem examination revealed marked pallor of the liver but there was no evidence of haemorrhage or marked loss of weight. Sternal marrow was macroscopically pink in colour but did not provide satisfactory smears.

Animals Transfused after 600 rads Irradiation

WBC Levels In figs. 7a, 7b and 7c the total WBC, neutrophil and lymphocyte levels in peripheral blood are shown at regular intervals up to 29 weeks after bone marrow transfusion. The general trends are similar to those found in animals transfused after 720 rads irradiation. A profound leucopoenia involving both lymphocytes and granulocytes develops by the 7th day after transfusion. The

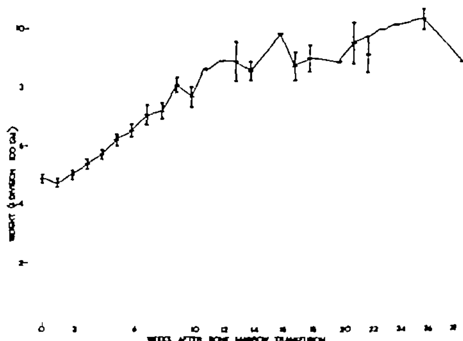


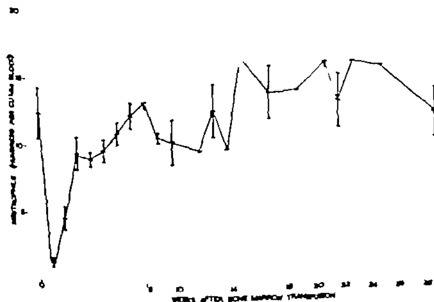
Fig. 6. Changes in total body weight of guinea-pigs transfused after 720 rads whole body gamma irradiation.

subsequent recoveries in the blood levels of the two types of leucocyte contrast sharply. The neutrophils are quite rapidly restored, the recovery commencing after the 1st week. Almost normal values are reached by the 3rd week and they remain only just below the normal range over the next 3 weeks. From the 6th week onwards their levels are within normal limits. In comparison, the recovery of blood lymphocytes is much more protracted. Their levels rise only very slowly and do not reach the normal range until the 10th week. In subsequent observations no marked changes in *lymphocyte* counts were noted.

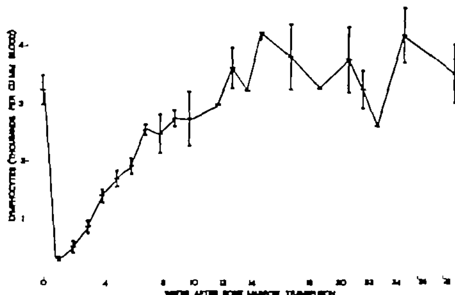
RBC and Reticulocyte Levels. The red cell counts of the transfused animals show a small depression for the first two weeks after transfusion (fig. 8a). Minimal values were recorded at the end of the 2nd week, when the levels had fallen from 5 116 000 ± 109 000 to 4 095 000 ± 360 000. Recovery however soon followed, the levels being almost normal by the end of the 3rd week and were finally restored by the 4th week. The red cell count thereafter re-

7a

Fig. 7 a, b, c. Changes in the total WBC, neutrophil and lymphocyte levels of peripheral blood in guinea-pigs transfused after 600 rads whole body gamma irradiation.



7b

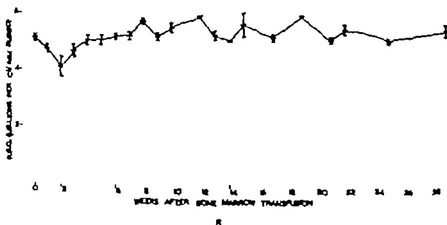


7c

mained within normal limits. Blood reticulocytes were considerably reduced by the end of the 1st week but this was immediately followed by a marked reticulocytosis, maximum levels being recorded on the 14th day (fig 8b) This reticulocytosis was more prolonged than that occuring in animals transfused after 720 rads, and a return to normal did not occur until 4 weeks after its onset. Subsequent levels remained within essentially normal limits.

Body Weight. There was no significant loss of weight during the 1st week after transfusion, but the weight-gain normally associated with young guinea pigs was not re-established until the 2nd week (fig 9) Thereafter body weight increased slowly but progressively to reach levels equal to those of normal adult guinea pigs.

Long-term Survival. At the time of writing 3 of the 10 animals transfused after 600 rads whole body irradiation still survive. Each of these has now lived for more than 3 years after transfusion, and this is comparable with the life-expectation of the normal guinea-pig (47) The ages at death of the transfused guinea-pigs which succumbed are 9½ months, 1 year 9 months (2 animals) 1 year 11 months, 2 years 1 month, 2 years 3 months, and 2 years 10



8

8b

Fig. 8a, b. Changes in the RBC and reticulocyte levels of peripheral blood in guinea-pigs transfused after 800 rads whole body gamma irradiation.

months. In only one animal was there post mortem evidence of a disturbance of haemopoiesis at the time of death. The animal died 2 years 10 months after transfusion and there was marked anaemia of the tissues with considerable body wasting. Peripheral blood examination 2 weeks before death showed anaemia (RBC 1 890,000

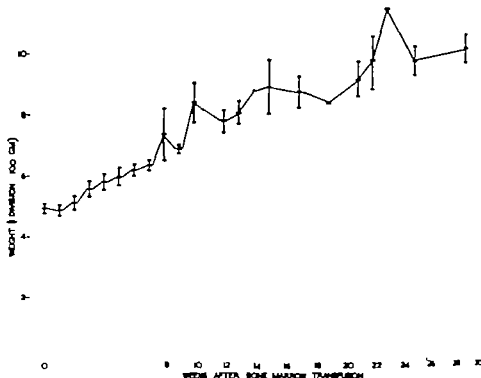


Fig 9. Changes in total body weight of guinea-pigs transfused after 600 rads whole body gamma irradiation.

per c.mm) but there was no leucopoenia (WBC 6,400 per c.mm) The differential white cell count was normal, platelets were present and also a very small number of reticulocytes.

Unusual Forms of Leucocytes Appearing in the Peripheral Blood of Transfused Animals

Differential counts of cells in smears prepared from the blood of transfused animals during the early weeks which followed bone marrow transfusion, revealed the presence of small numbers of unusual forms of leucocytes in the peripheral blood. These cells are not normally seen in the blood of young guinea pigs. Examples of cells of this type, observed within two weeks of bone marrow transfusion, are illustrated in fig 10 In the animals in which they occured, their mean percentage of the total WBC was 0 6 %, and the range from 0.2—2.8 %. Their mean absolute level was 9 cells per

c.mm, and the range from 1—31 per c.mm blood. These cells are rounded or oval in shape, and many are of considerable size, with diameters ranging between 17 and 22 μ. In one extreme case, the cell dimensions were 27 ×12 μ. In a minority of the cells, the diameter was between 13 and 16 μ. Their classification presents some difficulties. Many have the appearance of blast cells, some resembling monoblasts whilst other resemble lymphoblasts. Reticulum-cell features were evident in some cells. Cells in the smaller size-range resembled the so-called transitional cells found in guinea pig bone marrow (29 51)

Discussion

The Efficacy of the Transfused Bone Marrow

The present experiments show that bone marrow which itself is in the early stages of final regeneration following irradiation, can effectively protect severely irradiated guinea pigs. The weight-gain of these animals was only slightly checked, and their normal growth pattern was soon resumed. All of the transfused animals reached maturity and in the majority their life-span compared favourably with that of the normal guinea pig. Only one animal died with evidence of haemopoietic disorder there being a marked anaemia but there was no leucopoenia. Wasting had occurred with a considerable loss of depot-fat. Since this animal survived for nearly 3 years after transfusion its death could have been due to normal senility

Transfusion of irradiated bone marrow has been reported by several workers, their studies being made for various reasons. In no instance was the composition of the transfused marrow described. In the experiments of Meier and Brown (38) bone marrow was exposed to large doses of irradiation in vitro before it was transfused. Isologous and homologous irradiated marrows gave less protection than normal marrow and irradiated heterologous marrow actually increased the mortality rate in the transfused animals. Berman and Kaplan (3) investigated bone marrow for a factor which produced thymic regeneration. In these studies one group of animals received irradiated bone marrow which successfully protected them against lethal irradiation but did not produce thymic regeneration. In the experiments of Jacobson et al. (34) bone marrow was transfused 24 hours after it had been irradiated, and a

critical level of irradiation was found above which the transfused irradiated marrow appeared to be toxic to the recipients. A small survival rate was also noted after the injection of pooled marrow obtained from donors who 8 day previously had received 600 r

Maintenance of the Red Cell Count at High Levels after Transfusion

In the transfused animals, the peripheral RBC counts were sustained at surprisingly high levels. This was particularly so in animals transfused after 720 rads, in whom the red cell depression was only just detectable. The red cell counts appear to be sustained at high levels due to two factors a) a prompt recovery of erythropoiesis in the transfused animals, b) the relatively long life-span of the guinea pig red cell. A wave of erythropoietic recovery soon after transfusion was to be expected, if the pattern of regeneration remains the same as that which normally occurs when the marrow is left in situ in the donor (27) Evidence of this recovery was provided by a rapid and marked reticulocytosis in the peripheral blood during the 2nd week after transfusion.

The Early Recovery of Blood Neutrophil Levels

The early recovery of the blood neutrophils contrasts markedly with the protracted recovery of blood lymphocyte levels. In animals transfused after 720 rads, the blood neutrophils were restored to normal limits by the 14th day after transfusion.

Protracted Recovery of Blood Lymphocytes Despite High Lymphocyte Levels in Transfused Marrow

A marked lymphopoenia persisted for a considerable time in the transfused animals and normal levels did not return until 8-10 weeks after transfusion. When irradiated animals are transfused with normal bone marrow their lymphoid tissue also recovers (15, 42) and it is colonised by cells from the donor marrow (22) Its recovery is slower than that of bone marrow (37) but it may be speeded by injecting splenic cells (16) In the present studies, the prolonged lymphopoenia seems incongruous in view of the very high lymphocyte levels in the transfused marrow and especially since there is evidence of substantial lymphocyte production in this type of marrow (30) The apparent disparity may be explained in

elocytic series did not appear to be directly responsible for protection. BROWN et al (8) also concluded from transfusions involving excessively erythropoietic and granulopoietic marrows that a more primitive cell appears to be the essential stem-cell.

Fat in the Transfused Marrow Fat is grossly visible in the transfused marrow suspension. Fat storing reticulum cells are seen frequently in bone marrow at this stage of regeneration after irradiation (30) and the increase in fat causes a higher incidence of damaged cells in marrow smears (29) Only one animal died shortly after transfusion with fat laden marrow and it showed histological evidence of fat-embolism. In most cases the fine emulsification of the fat during suspension of the marrow appeared to preclude embolism. Retention of fat in the marrow transfusion may in fact, have been beneficial, since yellow bone marrow contains factors which stimulate haemopoiesis (35)

Significance of Unusual Forms of Leucocyte in the Blood of Transfused Animals

There is evidence that a small fraction of leucocytes in the blood of normal (7) and irradiated animals (6, 31) can synthetize DNA. Moreover amongst normal (41) and leukaemoid (46) leucocytes there is a stem-cell group which can protect against lethal irradiation. These cells also promote red cell regeneration, and thus support the monophyletic theory of haemopoiesis. In the present studies, the unusual forms of leucocyte circulating in the blood of the transfused animals shortly after marrow transfusion might be interpreted as stem-cells since some of them have blast characteristics. However they certainly are not found in the original transfusion Some are particularly notable for their large size and might arise as the result of initial irradiation of the donor marrow However giant granulocytes were never seen in the recipients blood. A few of these unusual leucocytes did resemble the larger type of transitional cell found in bone marrow (fig 10 g and h)

Acknowledgements We are very grateful for the support of Dr E. K. BLACKBURN in these studies also to Dr F E. NEAL and the Physicists of the Sheffield Regional Medical Physics Department for their help in lethal-dose studies and irradiation of guinea-pigs. We also thank Miss M. TURK and Miss C. HOLTBY for technical assistance.

Summary

Young guinea-pigs were successfully protected against the effects of lethal irradiation by injections of bone marrow which itself was in the early stages of foetal

several ways. Firstly the number of lymphocyte precursors in the transfusion may have been inadequate However the animals thrived after transfusion and there was no evidence of secondary disease which is associated with lymphoid tissue atrophy A further possibility is that the properties of bone marrow lymphocytes may be different from those produced in lymph nodes, and although these cells are produced in the marrow they may be retained there. Thus, lymphocytes contained in bone marrow transfusions may have only a weak ability to colonise lymph nodes, and may preferentially pass to the recipient's bone marrow for a specific purpose. Moreover if lymphocytes are discharged from the marrow into the blood stream they may be the type which has only a short intravascular life-span (40) and a lymphopoenia may persist until sufficient numbers of those lymphocytes which have a much longer intravascular span have accumulated in the blood. Finally there is evidence (28, 32) that blood lymphocyte levels do not always reflect the underlying activity in lymphoid tissues and bone marrow These levels will be determined by the balance between two dynamic processes, these being the entrance of lymphocytes into the circulation, and their exit from it. After lethal irradiation, the general depletion of lymphocytes throughout the body tissues must be very considerable. If, during regeneration, these cells are pouring into the tissues at a faster rate than they are entering the blood stream, then a lymphopoenia might persist.

The Unusual Composition of the Transfused Marrow

In the present experiments the transfused marrow gave effective protection but its cells differ markedly from those in normal bone marrow Three features must be stressed concerning its unusual composition a) between 70–80 / of its cells are mononuclear forms and are classified in the lymphoid series, b) only very small percentages of erythroid and granulocytic cells are present when compared with normal marrow and the levels of their early forms are particularly low c) the fat content is considerably raised when compared with that in normal red marrow

Types of Mononuclear Cells in the Transfusion. Mononuclear cells formed the great bulk of the transfusion. They may be sub-divided into two groups. The largest group (50–70 / of the total transfused cells) consist of typical small and medium-sized lymphocytes. The

elocytic series did not appear to be directly responsible for protection. Brown et al. (8) also concluded from transfusions involving excessively erythropoietic and granulopoietic marrows that a more primitive cell appears to be the essential stem-cell.

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Acta haemat. 32 168–182 (1964)

Aus der Medizinischen Universitätsklinik Zürich
(Direktor: Prof. P. H. Rossier)

Paroxysmale nächtliche Hämoglobinurie: In vitro- und in vivo Untersuchungen über die Beeinflussbarkeit der Hämolyse mit einer Mercaptanverbindung (Penicillamin)

Von A. von Felten

Obschon man das Krankheitsbild der paroxysmalen nächtlichen Hämoglobinurie (PNH) Ende des letzten Jahrhunderts (34) erstmals erkannt und seither in zahlreichen klinischen und experimentellen Untersuchungen von den verschiedensten Seiten her beleuchtet hat, ist bis heute weder seine Aetiologie noch seine Pathogenese vollständig geklärt. Allgemein gilt jedoch als gesichert (3, 5), daß bei der PNH ein erworbener Defekt der Erythrocyten vorliegt, welcher diese auf ein normalerweise im Serum vorhandenes hämolytisches System empfindlich macht. Dies zeigt sich darin, daß Patientenerythrocyten, die einer gesunden Person injiziert werden, eine verkürzte Lebensdauer aufweisen (7), umgekehrt jedoch Erythrocyten eines gesunden Spenders im Kreislauf des Patienten normal überleben (25, 16). Entsprechend verhält es sich mit den Hämolysetests in vitro, wie sie in der Diagnostik angewandt werden. Die Patientenerythrocyten lösen sich im eigenen wie auch im Serum gruppengleicher Normalpersonen auf, umgekehrt erfahren Normalerythrocyten im Patientenserum keine Hämolyse. Die bis heute bekannten Einzelheiten über die Natur des erythrocytären Defektes lassen sich noch nicht zu einem einheitlichen Bild zusammenfügen (5).

Auch die Frage nach dem *hämolytischen System*, das gegen die PNH-Erythrocyten zur Wirkung kommt, ist noch keineswegs beantwortet. Seit langem ist zwar bekannt, daß die hämolytische Aktivität des Serums durch Erhitzen auf 56 °C während 30 Minuten zerstört wird, andererseits aber diese thermolabile Komponente nicht mit dem Komplement identisch sein kann, da die Zugabe von

frischem Meerschweinchenserum zu keiner Reaktivierung des hämolytischen Systems führt (6, 11 35)

Mit der Entdeckung des Properdins (29 30) einer makromolekularen Eiweißkomponente im Serum, ist hiezu ein wichtiger Beitrag geleistet worden. HINZ et al. (14 15) haben nämlich nachgewiesen, daß ein properdinfreies Serum, welches alle Komponenten des Komplements enthält, die Fähigkeit zur Hämolyse von PNH Erythrocyten verloren hat erst nach Zugabe von Properdin tritt wieder eine Hämolyse auf. Umgekehrt ist ein properdinhaltiges Serum, welchem eine der Komplementkomponenten fehlt, ebenfalls nicht mehr hämolyseaktiv Durch Erhitzen des Serums auf 56 C während 30 Minuten werden sowohl das Properdin als auch das Komplement inaktiviert.

Der Properdinkomplex – er umfaßt das Properdin, die Komplementkomponenten sowie Magnesiumionen – soll bei der Abwehr gegen Bakterien und der Neutralisation von Viren eine entscheidende Rolle spielen. Die Natur des Properdins und besonders dessen Stellung gegenüber dem übrigen, immunologischen Abwehrsystem sind zwar immer noch Gegenstand eifriger Diskussionen, welche jedoch für die vorliegende Fragestellung nicht entscheidend ins Gewicht fallen dürften (18 26 27 28 33)

Bezüglich der chemischen Struktur gehört das Properdin zu den Makroglobulinen PILLEMER et al. (30) haben mittels Ultrazentrifuge eine Sedimentationskonstante von 27 S, ISLIKER UND LINDER (20) eine solche von 23–25 S bestimmt. Von DEUTSCH UND MORTON (8, 9) wurde erstmals gezeigt, daß Makroglobuline durch Einwirkung von Mercaptanverbindungen gespalten werden können. Bei der Spaltung werden die innerhalb des Moleküls vorhandenen Disulfidbrücken gesprengt und in freie SH-Gruppen übergeführt, wobei das Makroglobulin in kleinere Bruchstücke zerfällt und seine spezifische Funktion verliert. In der Folge ist dieses Verhalten bei den verschiedensten Makroglobulinen nachgewiesen worden, wobei besonders zu erwähnen ist, daß ISLIKER sowie HITZIG UND ISLIKER auch Properdinpräparate untersucht haben (Tabelle IV)

Für uns stellte sich nun die Frage, ob über eine Verminderung der Properdinaktivität eine symptomatische Beeinflussung der PNH, wenigstens während der akuten Hämolyseschübe, erzielt werden könnte dies umso mehr als im D-Penicillamin (β β-dimethyl-cystein) eine gut haltbare, praktisch nicht toxische Mercaptanverbindung für therapeutische Zwecke zur Verfügung steht.

In einer Kontrollserie verwendeten wir Serum, welches nicht mit Penicillamin, sondern durch Erhitzen auf 56 °C während 30 Minuten inaktiviert worden war.

Als Beispiel für die unter b) vorgenommenen Untersuchungen wird in Tabelle III ein Versuchsprotokoll angeführt.

Tabelle II

Hemmung der Hämolyse im Säuretest durch Zugabe von Penicillamin.

Serum	Penicillamin µg/ml Serum	Erythrocyten	pH vor Inkubation	pH nach Inkubation	Hämolyse
Kontrollperson 1	0	Patient 1	6,7	7,1	+++
Kontrollperson 1	5,0	Patient 1	6,8	7,1	0
Kontrollperson 1	4,0	Patient 1	6,7	7,1	+
Kontrollperson 1	3,0	Patient 1	6,8	7,2	++
Kontrollperson 1	2,0	Patient 1	6,7	7,2	+++
Kontrollperson 2	0	Patient 1	6,8	7,0	++
Kontrollperson 2	3,0	Patient 1	6,8	7,0	0
Kontrollperson 2	2,0	Patient 1	6,6	6,9	++
Patient 1	0	Patient 1	6,5	7,2	+++
Patient 1	5,0	Patient 1	6,7	—	0
Patient 1	2,5	Patient 1	6,7	—	+++
Kontrollperson 3	0	Patient 2	6,64	6,94	+++
Kontrollperson 3	2,0	Patient 2	6,64	6,81	0
Kontrollperson 3	1,5	Patient 2	6,66	6,89	+
Kontrollperson 3	1,0	Patient 2	6,65	6,91	+++
Kontrollperson 4	0	Patient 2	6,84	7,11	+++
Kontrollperson 4	2,5	Patient 2	6,71	7,02	0
Kontrollperson 4	1,5	Patient 2	6,72	7,01	+++
Patient 2	0	Patient 2	6,64	6,94	++
Patient 2	2,0	Patient 2	6,64	6,81	0
Patient 2	1,5	Patient 2	6,66	6,89	+
Patient 2	1,0	Patient 2	6,65	6,91	++
Kontrollperson 5	0	Patient 3	6,68	7,10	+++
Kontrollperson 5	2,5	Patient 3	6,74	6,96	0
Kontrollperson 5	1,0	Patient 3	6,71	6,93	+ (+)
Kontrollperson 5	0,5	Patient 3	6,50	6,69	+++
Kontrollperson 6	0	Patient 3	6,72	6,92	+++
Kontrollperson 6	2,0	Patient 3	6,73	6,91	0
Kontrollperson 6	1,0	Patient 3	6,60	7,03	+ (+)
Kontrollperson 7	0	Patient 3	6,50	6,88	++
Kontrollperson 7	2,0	Patient 3	6,44	6,97	0
Patient 3	0	Patient 3	6,54	6,95	+++
Patient 3	2,5	Patient 3	6,73	6,90	0
Patient 3	1,5	Patient 3	6,77	6,98	+++

In vivo-Untersuchungen

A. *Perorale Penicillaminapplikation über längere Zeit*

Patient 1 Zuerst wurden während 14 Tagen 1,5 g Penicillamin pro Tag gegeben (3 Kapseln um 14 Uhr 3 Kapseln um 18 Uhr 4 Kapseln um 22 Uhr) Anschließend verabreichten wir während weiterer 16 Tage 1,95 g pro die (3 Kapseln um 16 Uhr 3 Kapseln um 18 Uhr 3 Kapseln um 20 Uhr 4 Kapseln um 22 Uhr um hohe nächtliche Medikamentkonzentrationen zu erreichen) Die Patientin klagte über starkes Magenbrennen nach der Penicillamineinnahme, weshalb große Mengen Antacida gegeben werden mußten. Zudem trat am 11 Tag ein generalisiertes kleinfleckiges Exanthem auf, welches mit großer Wahrscheinlichkeit auf das Medikament zurückzuführen war Auf Antihistaminica besserte sich der Zustand so weit, daß das Penicillamin nicht abgesetzt werden mußte.

Am 14. und 18. Tag wurde jeweils 1 3 und 5 Std. nach der letzten Penicillamingabe um 22 Uhr am 30. Tag lediglich 5 Std. nachher eine Blutentnahme vorgenommen. Mit dem daraus gewonnenen frischen Serum wurde der Säure Hämolysetest durchgeführt.

Patient 2 und 3: Wir verabreichten beiden Patienten ambulant während 10 Tagen 1,95 Penicillamin pro Tag (4 Kapseln nach dem Morgenessen, 5 Kapseln nach dem Mittagessen, 4 Kapseln nach dem Abendessen) Am 10. Tag wurde, ca. 5 Std. nach der Mittagsdosis, eine einmalige Blutentnahme gemacht und in der gleichen Weise wie bei der 1 Patientin untersucht. Bei beiden Patienten traten keine Nebenerscheinungen auf.

B. *Kurzversuche mit intravenösen Penicillamininfusionen*

Das in den Kapseln enthaltene Penicillaminpulver wurde in Aqua dest. aufgelöst, keimfiltriert und direkt vor der Verwendung mit einer neutralisierenden Menge steriler Natronlauge versetzt*. Nachdem Voruntersuchungen gezeigt hatten, daß auf Injektion dieses Präparates weder eine pyrogene Reaktion noch eine andere Nebenwirkung auftrat, wurden 6 g Penicillamin mit 5%iger Glukose auf 600 ml ergänzt und über 5 Std. den Patienten 2 und 3 intravenös verabreicht; bei beiden zeigten sich keinerlei Nebenwirkungen.

Zur Durchführung der Säure-Hämolysetests nahmen wir zu folgenden Zeitpunkten eine Blutentnahme vor direkt vor Infusionsbeginn, am Schluß der Infusion sowie 1 3½ und 5½ Std. nach Infusionsende.

Resultate und Diskussion

In vitro-Versuche. Wie aus Tabelle II hervorgeht, konnte bei allen 3 Patienten der für die PNH pathognomonische Säure-Hämolysetest, welcher sowohl mit Eigenserum als auch mit verschiedenen gruppengleichen Kontrollseren durchgeführt wurde, durch vorausgehende Inkubation des Serums mit Penicillamin gehemmt werden. Der Grad der Hemmung ist von der Penicillaminkonzentration abhängig Die Kontrollen mit physiologischer

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* Die Herstellung der Infusionslösungen sei an dieser Stelle der Kantonsapotheke des Kantonsspitals Zürich bestens verdankt.

Kochsalzlösung sowie die pH Messungen zeigen, daß diese Hemmung weder als Verdünnungseffekt noch mit einer Änderung des pH durch das Penicillamin zu erklären ist. Damit scheint sich die Arbeitshypothese, welche zu Beginn dargelegt wurde, bestätigt zu haben. Insbesondere ist darauf hinzuweisen, daß die zur Hemmung benötigte Penicillaminmenge mit den in der Literatur angegebenen Konzentrationen zur Spaltung der Makroglobuline übereinstimmt (Tabelle IV).

Da es uns nicht möglich war das Properdin direkt zu bestimmen, versuchten wir mit weiteren Untersuchungen wenigstens indirekt diese Theorie zu untermauern.

Vorerst wurde die Möglichkeit einer gleichzeitigen Zerstörung des Komplements in Erwägung gezogen, besonders auch da Lino (24) eine Abnahme des Komplementtiters während einer peroralen Penicillamintherapie bemerkt hatte. In unseren Kontrolluntersuchungen vermochten nun alle Seren, welche mit der zur Hämolysehemmung notwendigen minimalen Penicillaminmenge über 2 Std. behandelt wurden, sensibilisierte Schaferythrocyten vollständig aufzulösen, nur wenn die Seren mit größeren Penicillamindosen versetzt und während 3 Stunden bei 37° C inkubiert wurden, trat eine partielle Hemmung der Lyse von Schaferythrocyten ein. Im weitern wurde im Säure-Hämolysetest die mittels Penicillamin erzielte Hemmung durch Zugabe von Meerschweinchenserum nie behoben (Tabelle III). Ein Komplementmangel als Ursache der Hämolysehemmung ist damit auszuschließen.

Die Inaktivierung des Properdinkomplexes könnte durch ein Fehlen der notwendigen Magnesiumionen bedingt sein, auch diese Möglichkeit fällt außer Betracht, da die Zugabe von Magnesiumionen in verschiedenen Konzentrationen (1/1000 bis 1/6 n) mit und ohne Komplementbeimengung ebenfalls zu keiner Reaktivierung des Hämolysesystems führte.

Aus der Arbeit von Pillemer (30) geht hervor, daß Rattenserum ausgesprochen properdinreich ist (25–30 E/ml), Meerschweinchenserum dagegen nur wenig Properdin enthält (1–2 E/ml). Pillemer erwähnt auch, daß Meerschweinchenserum gegen PNH Erythrocyten inaktiv sei, nach Zugabe eines Properdinpräparates oder von Rattenserum aber eine Hämolyse auftrete. An dieser Stelle soll zudem ein Fall von PNH erwähnt werden, den Blaisdell et al. (1) beschrieben haben. Es fand sich bei diesem Patienten ein negativer Säure-Hämolysetest mit Eigenserum, während er mit

einem Kontrollserum positiv ausfiel als Erklärung dafür ergab die weitere Untersuchung einen verminderten Properdingehalt bei normalem Komplementtiter im Serum des Patienten. Bei Zugabe von Meerschweinchenserum blieb der Test weiterhin negativ hingegen konnte das Serum mittels eines gereinigten Properdinpräparates aktiviert werden. In unseren Experimenten verhielt sich das mit Penicillamin behandelte Serum wie das oben erwähnte Serum mit nachgewiesenem Properdinmangel Ein Zusatz von Meerschweinchenserum führte zu keiner Aktivierung (bzw mit großen Mengen trat, entsprechend der geringen Zufuhr von Properdin, eine schwache Hämolyse auf) Im Gegensatz dazu zeigte sich mit Rattenserum je nach zugesetzter Menge eine mäßige bis deutliche, mit der Aktivität im Nativserum gleichzusetzende Hämolysierung der PNH Erythrocyten, welche durch gleichzeitige Zugabe von Meerschweinchenserum nicht mehr gesteigert werden konnte.

In der Kontrollserie, bei welcher das Serum durch Erhitzen auf 56 °C während 30 Minuten inaktiviert wurde, erwies sich die Reaktivierung der Hämolyse durch Rattenserum als viel geringer (Tabelle III) Dies könnte darauf hindeuten, daß der hitzelabile Anteil des hämolytischen Systems außer Komplement und Properdin noch weitere Komponenten umfaßt die durch Penicillamin nicht beeinträchtigt, wohl aber durch Wärme vernichtet werden zudem lassen sie sich offenbar weder mit Meerschweinchen noch mit Rattenserum genügend ergänzen.

In vivo-Untersuchungen. Nach *peroraler* Penicillaminapplikation ließ sich bei keinem der 3 untersuchten Patienten im Säure Hämolysetest eine vollständige oder auch nur partielle Hemmung der Hämolyse von Patientenerythrocyten nachweisen. Es wurde zudem versucht zu ermitteln, ob die vor der Therapie bestimmte minimale hämolysehemmende Penicillamindosis im Patientenserum nach der Penicillaminbehandlung immer noch gleich hoch sei. Auch hier zeigte sich bei Patient 2 und 3 keine Dosisverminderung lediglich bei der 1 Patientin konnte in der zweiten Untersuchung (18 Tage nach Therapiebeginn, 4 Tage nach 1 95 Penicillamin pro die) eine Stunde nach der letzten Penicillaminverabreichung bereits durch Zugabe von 2,5 mg Penicillamin/ml Patientenserum in vitro eine zwar unvollständige, aber doch signifikante Verminderung der Hämolyse festgestellt werden. Vor der peroralen Penicillamintherapie war mit Patientenserum unter Zugabe der gleichen Dosis nie eine Beeinflussung der Hämolyse erreicht worden. Klinisch zeigte sich

auch bei dieser Patientin während der 30tägigen Penicillaminverabreichung keine Beeinflussung des Krankheitsbildes. Der konstante Hämoglobinabfall, der immer wieder durch Erythrocytentransfusionen behoben werden mußte, hielt während dieser Zeit unvermindert an.

Um wenigstens über kurze Zeit eine höhere Plasmakonzentration zu erreichen, wurden in zwei Versuchen je 6 g Penicillamin während 5 Stunden *intravenös infundiert*. Auch damit konnte im Säure-Hämolysetest keine Wirkung festgestellt werden. Insbesondere erfuhr die für die Hemmung in vitro benötigte Penicillaminzugabe keine Verminderung.

Unsere Untersuchungen zeigten somit eine deutliche Diskrepanz zwischen den positiven Resultaten in vitro und dem vollständigen Fehlen jeglicher Wirksamkeit in vivo. Wenn man die Penicillaminmenge, die zur Hemmung der Hämolyse in vitro notwendig ist, in Rechnung setzt, mußten eigentlich diese negativen Resultate erwartet werden, da in vivo niemals eine vergleichbare Konzentration zu erreichen war. Die perorale Therapie ergab, wenn man die ganze Tagesdosis auf das totale Plasmavolumen der Patienten umrechnet, nie eine Konzentration von 1 mg/ml. Auch bei intravenöser Verabreichung dürfte dieser Penicillamingehalt nicht überstiegen worden sein, wenn man den Übergang in den extravasalen Bereich sowie den fortwährenden Abbau und die rasche Ausscheidung berücksichtigt.

Dieselbe Problematik zeigt sich bei der Durchsicht der Literatur über in vitro- und in vivo-Untersuchungen mit Penicillamin bei anderen Krankheiten (Tabelle IV und V). Eine Ausnahme davon machen nur die Arbeiten von Ritzmann (31, 32) und Jaffe (21). Ritzmann konnte sowohl bei Patienten mit einer Kälteagglutininkrankheit als auch mit einem Morbus Waldenström klinisch und in den Laboruntersuchungen nach peroraler Applikation von Penicillamin eine deutliche Beeinflussung der Krankheiten nachweisen, obschon hier dieselbe Diskrepanz zwischen den für die in vitro-Versuche benötigten Mengen und der in vivo verabreichten Dosis bestand. Es ist vielleicht von Bedeutung, daß in diesen Fällen ein DL-Penicillamin verwendet wurde. In den Untersuchungen von Jaffe konnte bei einem Patienten mit primär chronischer Polyarthritis nach mehrwöchiger Verabreichung von D-Penicillamin durch Wechseln auf DL-Penicillamin eine nochmalige Verminderung des Rheumafaktors erreicht werden. Somit

Tabelle 1
Spaltung von Makroglobulinen mit Mercaptan-Verbindungen. In vivo-Untersuchungen.

Autor	Makroglobulinart	Mercaptan-Verbindung und Dosierung	Resultate klinisch	Resultate in
Hitzig u. Isliker (17)	M. Waldenström	Cysteamin, intravenös	ø	keine Veränderung in der Ultrazentrifuge leichte Viskositätsabnahme
Bloch et al. (2)	M. Waldenström	DL-Penicillamin, 1,0 g/Tag 5 Tage lang	ø	Abnahme der γ-Globuline in der Papierelektrophorese
		DL-Penicillamin 1,0 g/Tag 5 Tage lang, anschließend während 9 Tagen 2,0 g pro Tag	ø	Abnahme der γ-Globuline in der Papierelektrophorese
Rittmann und Levin (32)	Kälteagglutininkrankheit	DL-Penicillamin 1,5 g/Tag während 10 Tagen	++	deutliche Abnahme des Kälteagglutinintiters verzögerter Abbau der Erythrocyten (Chromkurve)
Rittmann et al. (31)	M. Waldenström	DL-Penicillamin 1,0 g/Tag während 19 Tagen	++	ab 2. Therapietag Abnahme der Viskosität am 6. Tag Aufsplitterung der γ-Globulinzacke in der Papierelektrophorese, keine Gellerung des Serums mehr in Kälte Sia-Test negativ geworden
Jaffe (21)	Prim. chron. Polyarthritis	DL-Penicillamin 2 g/Tag 6 Wochen lang	ø	Abnahme des Rheumafaktors in der 4. Woche; nach Absetzen der Therapie während weiterer 4 Wochen vermindert.
		D-Penicillamin 2 g/Tag 10 Wochen lang, anschließend DL-Penicillamin 2 g/Tag für weitere 5 Wochen	ø	beginnende Verminderung des Rheumafaktors nach 2 Wochen; weiterer Abfall auf DL-Penicillamin; nach Therapie noch während 10 Wochen herabgesetzt.
Lamm et al. (24)	Kälteagglutininkrankheit	D-Penicillamin 3,0 g/Tag während 11 Tagen	ø	keine Veränderung in der Ultrazentrifuge, keine Abnahme des Kälteagglutinintiters.
		D-Penicillamin 1,5 g/Tag + Probenecid 1,0 g/Tag während 7 Tagen	ø	keine Veränderung in der Ultrazentrifuge, keine Abnahme des Kälteagglutinintiters.

ist nicht ausgeschlossen daß die L-Form auch bei RITTMANN zur besseren Wirksamkeit beigetragen hat. Das L-Penicillamin wird jedoch heute wegen seiner toxischen Nebenwirkungen (22, 23 36) nicht mehr angewandt.

Zum Schluß sei noch die Frage aufgeworfen, ob mit einer Verminderung des Properdins im Plasma von Patienten mit PNH der hämolytische Prozeß in vivo wirklich entscheidend zu beeinflussen ist. In diesem Zusammenhang muß darauf hingewiesen werden, daß HINZ (15) keinen Verbrauch des Properdins unter der Hämolyse der PNH Erythrocyten feststellen konnte zudem wies der Patient von BLAISDELL (1) klinisch eine deutliche Hämolyse auf, obschon das Serum infolge des Properdinmangels im Säure-Hämolysetest inaktiv war Die Frage, inwieweit also der Hämolysemechanismus im Säure Hämolysetest als repräsentativ für den Hämolysevorgang in vivo gelten darf, kann demnach nicht sicher beantwortet werden.

Zusammenfassung

Im hämolytischen System, das gegen die pathologischen PNH-Erythrocyten zur Wirkung kommt, ist das Properdin, ein in jedem Serum vorhandenes Makroglobulin, maßgeblich beteiligt. Da Makroglobuline durch Mercaptanverbindungen gespalten und dadurch inaktiviert werden, stellte sich die Frage, ob auf diesem Wege die Hämolyse bei der PNH zu beeinflussen sei. In vitro-Versuche ergaben tatsächlich eine Hemmung des für die PNH charakteristischen Säure-Hämolysetests durch Penicillamin (Dimethylcystein) Dabei konnte ein Mangel an Komplement oder an Magnesiumionen, die zur Wirkung des Properdins nötig sind, ausgeschlossen werden. Mischversuche mit properdinreichem Ratten- bzw properdinarmem Meerschweinchenserum ließen darauf schließen, daß die Hämolysehemmung wirklich auf einer Zerstörung des Properdins beruhte. Die perorale und intravenöse Verabreichung von Penicillamin bei 3 Patienten mit PNH hatte hingegen keinen signifikanten Einfluß, da die hämolysehemmende Plasmakonzentration des Medikamentes in vivo nicht erreicht werden konnte.

Summary

Properdin is an essential factor of the hemolytic system affecting the erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (PNH). Since macroglobulins like properdin can be broken into smaller inactive components by mercaptanes, the possibility of influencing the hemolysis of PNH erythrocytes by penicillamine was investigated. The in vitro hemolysis in the acid-test was completely inhibited by penicillamine This result was obtained without influencing the activity of complement or magnesium ions which are both necessary for properdin activity The hemolytic system could be restored with properdin-rich rat serum but not with properdin-poor guinea-pig serum, thus suggesting that penicillamine inhibits hemolysis by inactivating properdin. The oral and the intravenous administration of penicillamine to three patients with PNH had no significant effect on the hemolysis because it was not possible to attain the drug levels effective in vitro

Résumé

La properdine, une macromolécule globulinique, est un facteur essentiel du système hémolytique, qui touche les érythrocytes des malades atteints d'une hémoglobinurie paroxystique nocturne (HPN). Comme les macroglobulines peuvent être modé par des mercaptanes dans des composés inactives, la possibilité d influencer l'hémolyse des érythrocytes HPN par la penicillamine été recherché. L'hémolyse in vitro en milieu acide pu être inhibé complètement. Ce résultat été obtenu sans influencer ni l'activité du complément, ni les ions de Mg, tous les deux nécessaires pour l'activité de la properdine. Le système hémolytique pu être rétabli avec du sérum de rat riche en properdine, mais pas ec du sérum de cobaye pauvre en properdine, ce qui suggère que la pénicillamine inhibe l'hémolyse en inactivant la properdine. L'administration orale et intraveineuse de pénicillamine à trois malades atteints de HPN n'est pas eu un effet sur l'hémolyse, comme on pas pu atteindre in h des taux médicamenteux assez élevés.

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meter of 8 mm, the height of the glass bead column thus being approximately 30 cm. 2 ml of heparinized blood, approximately 1 hour old, from the blood donor centre, was poured over the glass beads and was allowed to pass through 5.5 ml of these, after which the tap was closed. 1 minute after the start of the experiment the tap was re-opened and the blood was allowed to pass through a further 0.5 ml of glass pellets, the tap then being closed again. Each succeeding minute the blood was passed through 0.5 ml of "new" pellets in this way. At 9 minutes after the start the blood was let down to the 10 ml mark, and at 10 minutes it was released through the tap and the first three drops were taken for examination. The number of polymorphonuclear blood cells (mainly neutrophil granulocytes) and mononmorphonuclear blood cells (lymphocytes and monocytes) per cmm in the blood before and after its passage through the glass beads were counted in a Burker counting chamber (dilution 1:20, 2x 32 B squares counted). In addition the eosinophil granulocytes were counted in a Fuchs-Rosenthal chamber (dilution 1:20, the whole chamber counted). The number per cmm in the blood after passage was given as a percentage of the number per cmm before. Blood from 28 donors was examined (table I).

Experiment I B: This experiment was in the main performed in a similar manner to experiment 1 A, but the blood was passed through the glass beads more rapidly. When the blood had passed through 5.5 ml the tap was kept closed until 2 minutes after the start, when the blood was released through 1.5 ml of "new" beads. Each succeeding minute the blood was passed through a further 1.5 ml of beads, and 5 minutes at was re-leased through the tap. The first three drops were taken for examination in this experiment also, and the number of cells per cmm in the blood after passage was given as a percentage of the number per cmm before. Blood from 28 donors [illegible] examined (table I).

Results

As shown in table I the eosinophil granulocytes showed the greatest loss from the passage through the glass beads. The mononuclear blood cells adhered least and the neutrophil granulocytes took an intermediate position.

Table I

The passage of different types of blood cells in experiments 1 A and 1 B, given as percentages. Mean and error of the mean.

	Polynuclears	Mononuclears	Eosinophils
1 A	51.5 ± 3.6	80.2 ± 4.7	38.1 ± 4.3
1 B	61.9 ± 4.5	86.0 ± 4.5	45.2 ± 5.6

Differential Counts of Blood before and after the Passage of Glass Beads (Experiment 2)

Method

Blood from three donors was separated through 10 ml glass beads as according to the time scheme in experiment 1 A. The total number of white blood cells per cmm

was counted in Bürker counting chamber before and after the separation. Blood smears were prepared before and after the separation and 200 cells from each sample were differentially counted and the number of segments in the neutrophil granulocytes recorded. Sufficient blood for 4 separations was taken from each donor 2 separations being performed 1 hour after and 2 separations 4 hours after venesection.

Results

The differences in the passage capacity between the polynuclear mononuclear and eosinophil blood cells were confirmed in this small investigation. The percentage passages of the different types of blood cells were neutrophil granulocytes 67.9 eosinophil granulocytes 56.0, monocytes 83 7 and lymphocytes 70.9 Analysis of the number of segments in the neutrophil granulocytes before and after separation gave a similar percentage distribution of the different nuclear forms. The basophil granulocytes appeared to pass through without any noteworthy loss 0 4 / basophil before and 0.5 / after the separation. No difference between the separations after 1 and 4 hours was found.

The total Number of Polymorphonuclears Mononorphonuclears and Eosinophils before and after the Passage of Glass Beads (Experiment 3)

Method

Heparinized blood from 13 donors was passed through 10 ml of glass beads 1 and 2 hours after the venesection, as according to the time scheme used in experiment 1 A. Exactly 2 ml blood were poured down through the glass beads. At 10 minutes, when the tap was opened and the blood ran out, 2 ml of Earl' solution were added. The tap was left completely open. After further 5 minutes (15 minutes from the commencement of passage) the collected fluid was measured.

The total numbers of polynuclear mononuclear and eosinophil blood cells introduced among the glass beads and collected after their passage through them were counted as in experiment 1. The numbers that passed through were given as percentage of those that were introduced.

Results

As seen in table II the polynuclear blood cells and eosinophils were found in the washing fluid in approximately the same percentage, i. e. about 80 %, while almost all of the lymphocytes passed through in this experiment. No difference was observed between the runs at 1 and 2 hours after venesection.

Table II

The passage of different types of blood cells in experiment 3, given as percentages. Mean and error of the mean.

	Polynuclears	Mononuclears	Eosinophils
1 hour after venesection	78.3 ± 5.3	93.8 ± 13.2	79.5 ± 6.2
2 hours after venesection	81.4 ± 6.8	100.4 ± 7.6	82.5 ± 10.6

Discussion

A comparison between the results of experiments 1 and 3 indicates that the neutrophil blood cells adhering to the glass beads become relatively firmly attached while the mononuclear and eosinophil may be washed away fairly easily. In all of these investigations the error of the method is very large, since relatively few cells are counted in the Bürker counting chamber. The coefficient of variation in counting 2×32 B squares from different samples of one particular blood is approximately 10 %. The purpose of the experiment was to give only a rough estimate of the tendency of different white blood cells to adhere to glass.

Summary

A method of studying the trapping of white blood cells on glass beads is described. The eosinophils had the greatest tendency to adhere to the glass beads, followed by the neutrophil granulocytes, while the mononuclear blood cells exhibited the least tendency to adhere. The monocytes and lymphocytes had similar passage capacity. The basophil granulocytes appeared to pass over the glass beads without adhering. Granulocytes with rod-shaped and segmented nuclei had similar passage capacities. The storage of heparinized blood had no noteworthy effect on the percentage passage capacity. The neutrophil granulocytes adhering to the glass beads became firmly attached, while the eosinophil granulocytes and the mononuclear blood cells could be more easily washed out.

Résumé

Les auteurs présentent une méthode pour étudier l'attachement des globules blancs à des perles de verre. Les éosinophiles ont la plus grande tendance d'adhérer aux perles de verre, suivies par les granulocytes neutrophiles, tandis que les cellules sanguines mononucléaires ne montrent pas beaucoup de tendance d'adhérer. Les monocytes et les lymphocytes se comportaient d'une façon similaire. Les granulocytes basophiles semblaient à passer les perles de verre sans y adhérer. Les granulocytes segmentés et non segmentés avaient des capacités de passage semblables. La conservation de sang héparinisé n'avait pas un effet notable sur la capacité de passage. Les neutrophiles montraient un attachement ferme aux perles de verre, tandis que les éosinophiles et les cellules mononucléaires se laissaient plus facilement laver.

Zusammenfassung

Es wird eine Methode beschrieben zum Nachweis der Haftung weißer Blutzellen an Glasperlen. Eosinophile zeigten die größte Haftungstendenz, dann folgten die Neutrophilen, während mononukleäre Blutzellen die geringste Haftung aufwiesen. Dabei verhielten sich Monozyten und Lymphozyten ähnlich. Basophile Granulozyten schienen die Glasperlen zu passieren, ohne daran zu haften. Stabkernige und segmentkernige Granulozyten hatten eine ähnliche Passagefähigkeit. Die Aufbewahrung von heparinisiertem Blut hatte keinen nennenswerten Effekt auf die Passagefähigkeit. Neutrophile zeigten eine feste Haftung an den Glasperlen, während Eosinophile und mononukleäre Blutzellen sich leicht auswaschen ließen.

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Acta haemat. 32. 188–192 (1964)

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Fetal Haemoglobin in Acquired Aplastic Anaemia

By Muzaffer Aksoy and Fuzuli Seçer

The alkali resistent haemoglobin found in different haematologic disorders is most probably fetal haemoglobin. This has been established by different methods (1—4). As it is well known, fetal haemoglobin is found in some forms of congenital haemolytic anaemias, particularly in thalassaemic syndromes. On the other hand, it is generally assumed that small amounts of fetal haemoglobin may also be encountered in some acquired haematologic disorders, such as chronic and acute leukaemias (5—7), untreated pernicious anaemia (7), multiple myeloma (8), Di Guglielmo's disease (7) and molar pregnancy (9). Although Shorr et al. have found definitely elevated level of alkali resistent haemoglobin in arregenerative anaemia (5), the results of haemoglobin analyses in the patients with aplastic anaemia obtained by Beaven et al. were negative (6).

Recently Shahidi et al. made haemoglobin analyses in the patients with congenital and acquired haemolytic anaemias (10). Fetal haemoglobin varied from 3 to 15 % in 17 patients. In most patients the alkali resistent haemoglobin persisted long after the anaemia was corrected. Similar results were also obtained by Jonxis in the patients with constitutional apalastic anaemia, namely the amount of fetal haemoglobin ranged between 5 and 25 % (11)*.

Considering the conflicting results on the occurrence of fetal haemoglobin in aplastic anaemia we made a study of haemoglobin analysis in the patients with acquired aplastic anaemia.

Material and Methods

Our series consisted of 7 males and 3 females from 7 to 60 years of age. With the exception of one patient (case 5) they presented all the severe form of acquired aplastic

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* Recently we have performed haemoglobin analysis of a patient with constitutional aplastic anaemia. Fetal haemoglobin was 15%.

anaemia. They had all clinical manifestations and haematologic findings characteristic for aplastic anaemia. Some clinical data and haematologic findings are summarized in table I. In six patients, the cause of aplastic anaemia was not found. In the remaining four cases, benzene, sulfanilamide and novalgin were possibly the causative agents for the development of aplastic anaemia.

Fetal haemoglobin was determined by the method of Singer et al. (5). In some patients the method of Kleihauer and Betke was also used (12). Haemoglobin A_2 was determined by the method of discontinuous TRIS buffer paper electrophoresis according to the technique of Goldberg (13). The maximum normal values for haemoglobin A_2 in this was 3.5 %.

Results

As can be seen from table I, with the exception of two cases, all the patients with acquired aplastic anaemia showed a mildly or moderately elevated level of fetal haemoglobin. The amount of fetal haemoglobin ranged between 3.1 and 15 %. On the other hand, only in five patients the content of alkali resistent haemoglobin was above 10 %. In three patients, the level of fetal haemoglobin at admission was normal. But during the course, some months later (approximately 2 and 6 months) the amount of fetal haemoglobin increased and it reached to the level of 12.6, 13 and 15 % respectively.

Comment

Our study showed an increased level of fetal haemoglobin in the majority of the patients with acquired aplastic anaemia, namely in 80 % of the cases. These results are in accordance with those of Singer et al. (5) and Shahidi et al. (10). In addition to that, Jones (11) and Shahidi et al. (10) found an abnormally increased level of alkali resistent haemoglobin in the cases of constitutional aplastic anaemia, particularly in Fanconi syndrome.

The interesting point in this investigation was that although in some patients with acquired aplastic anaemia at admission the level of fetal haemoglobin was in normal range, some months later approximately 2 and 6 months, the amount of alkali resistent haemoglobin increased. For example, in case 2, a classical example of acquired aplastic anaemia due to benzene, the level of fetal haemoglobin at admission was 0 %. But, nearly two months later the amount of alkali resistent haemoglobin increased and reached to 15 %. The same happened in case 3, a case of acquired aplastic anaemia possibly due to sulfanilamide, the percentage of fetal haemoglobin at admission was nill and two months later alkali resistent haemoglobin was found as 4 %. On third examination,

Table I

Some haematologic findings and the results of haemoglobin analysis in 10 patients with acquired aplastic anemia.

Case no.	Sex	Age	Duration of illness	Possible etiology	Pancytopenia	Bone marrow	Treatment received	Hb. F on admission, %	Hb. F % during therapy	Hb. F % following therapy	Hb. A₂ %
1	F	58	8/12	Novalgin	++	Very hyper-cellular	Transfused several times. Anabolics		3.1		1.8
2	M	15	2/12	Benzene	++++	Normocellular	Transfused several times. Steroid + anabolic	0	15	0	2
3	M	42	3/12	Sulfanilamid	++++	Hypocellular	Transfused sever 1 times. Steroid + anabolics	0	4	12.6	2
4	M	32	4/12	Unknown	+++	Normocellular	Transfused several times. Steroid + anabolics	0	0	0	2.5
5	M	10	3	Unknown	++	Hypercellular	Transfused several times. Steroid + anabolics	0	0	0	2.5
6	F	27	5/12	Unknown	++	Hypocellular	Transfused sever 1 times. Steroid + anabolics		5.5		1.0
7	M	28	1	Desrol	+++	Normocellular	Transfused sever 1 times. Steroid		12.5		2.1
8	M	7	3/12	Unknown	+++	Hypocellular	Transfused several times. Steroid + anabolics		4.6		2
9	F	60	0/12	Unknown	++	Hypercellular	Transfused several times.		12.5		2.5
10	M	48	2	Unknown	+	Hypercellular Later acellular	Transfused several times. Steroid + anabolics	0	13		2

Not performed

when the patient was completely recovered from aplastic anaemia, the level of fetal haemoglobin was found as 12.6 %. Although in above mentioned two patients the clinical and haematologic improvement was nearly complete, the increase in the level of fetal haemoglobin persisted (despite this, there was a small drop in alkali resistent haemoglobin of one of them, case 2 following the recovery). This very interesting finding confirms the results of SHAHIDI et al. (10). In this connection it is worth to mention that the increase in fetal haemoglobin may precede the onset of pancytopenia which was established by SHAHIDI et al. in one patient with constitutional aplastic anaemia. On the other hand, as we have described above, the onset of frank pancytopenia in our three patients with acquired aplastic anaemia preced several months the increase in alkali resistent haemoglobin. This can be seen very clearly in table I. From this standpoint there is a difference between acquired aplastic anaemia investigated by the present authors and constitutional aplastic anaemia studied by SHAHIDI et al.

As regards the mechanism of the production of fetal haemoglobin in aplastic anaemie for the time being we are not able to answer this question properly. As it is well known, fetal haemoglobin almost completely disappeares in early childhood. But the synthesis of fetal haemoglobin at low level continues during adult life. Therefore, small amounts of fetal haemoglobin may be found in the blood of normal adults. This has been definitely shown by the method of alkali denaturation and immunologic studies (3—5). Considering the occurence of increased amount of fetal haemoglobin in aplastic anaemia, one may rightly speculate that there are some alteration in haemopoietic system which may stimulate the fetal haemoglobin production in increased amount in adult life.

Summary

An increased amount of fetal haemoglobin was found in eight of ten patients with acquired aplastic anaemia. In three patient, the onset of pancytopenia preceded several months the increase in alkali resistent haemoglobin. In two patients, although the clinical and haematologic improvement was nearly complete, the increase of fetal haemoglobin persisted.

Résumé

Une augmentation de l'hémoglobine foetale été trouvée chez 8 sur 10 malades d'une anémie aplastique acquise. Chez 3 malades le début de la pancytopénie se situait plusieurs mois avant l'augmentation de l'hémoglobine alcali résistante. Deux

malades montraient malgré une amélioration clinique et hématologique une persistance de l'hémoglobine foetale.

Zusammenfassung

Bei 8 von 10 Patienten mit erworbener aplastischer Anämie wurde eine Vermehrung von fötalem Hämoglobin gefunden. Bei 3 Fällen begann die Panzytopenie mehrere Monate vor dem Anstieg des alkaliresistenten Hämoglobins. Bei 2 Patienten blieb trotz klinischer und hämatologischer Besserung die Vermehrung von fötalem Hämoglobin bestehen.

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Acta haemat. 32, 193–199 (1964)

From the Pathological Institute, Hannover (Director Prof. Dr. med. O. Fresen)

The Submicroscopical Structure of the Reticular Cell Tissue

By O. Fresen

As the system of reticular cell tissue is not an organ in the conventional meaning but distributed in various forms in the body only a synoptic evaluation of individual experiments and pathologico-anatomical findings, proved by histological examination and electron microscopy, can lead to an understanding of its morphology.

The *structure of the reticular cell tissue* is fairly simple: it shows ramifying interconnected cells which in quiescence have a clear and unstructured plasma with an apparatus of Golgi near the nucleus. The relatively large and often lacunar nucleus contains little chromatin, but usually possesses a nuclear body and a distinct nuclear membrane. These cells are little characteristic interstitial cells which on light microscope examination give the impression of a syncytial configuration. In this cellular compound fibrils occur which are closely related with the exoplasm and form the spatially oriented network of the lattice fibres. They differ from the collagen fibres of ordinary connective tissue by their fineness, their property of tinging on plasma staining and their chracteristic although non-specific ability to take up silver. This fibrillary reticulum shows a uniform structure which determines the structure of lymph nodes, tonsils, spleen and bone marrow. As the cell stroma of the lympho-reticular tissue also outside the lymph nodes it harbours the lymphocytes and the myeloic blood cells in the reticulum of the bone marrow; in the spleen it forms the spongy blood-conducting pulp.

Electron-microscope studies confirmed that the reticular cell is a large, multibranched and only slightly characteristic cell which, according to its functional needs, exhibits a comparatively variable minute structure (4). The increase in mitochondria and ergastoplasm lamelles and vacuoles begins the transformation to active macrophages. In contradistinction to the light microscope impression of a syncytial structure in the submicroscopic range the

reticular cell tissue shows continuous cleft like cell borders which, because of the marked spatial interrelationship of the cells, form a bizarre system of joints (fig 1) The continuous intercellular clefts widen to intercellular spaces of very different shapes, up to 0.8 μ wide. In these clefts and lacunae run longitudinal and cross-striated fibrils. With a medium thickness of 200 to 400 Å they are not always sharply differentiated from the interfibrillary homogenous, or scarcely granulated ground substance. Their transverse striation shows a periodicity of about 610 Å splitting is not recognizable in them. Several fibrils run together either straight or slightly undulated, and separate around groups of equally ball-shaped formations. Cross sections show pale circular discs with a slightly densified margin about 60 Å wide. These fibrils together form an oriented, obviously non ramifying bundle reaching the dimension of a lattice fibre visible, when silverblackened by light microscopy This bundle has been confirmed to be the fine structure of the reticular fibres also by its differences from collagen fibre (8) seen on electron microscopy As these intercellular spaces, filled with fibres, are occasionally almost completely surrounded by the cytoplasm of one of the various reticular cells, they may give a false impression of being intracellularly situated. This appearance can be understood at first only as the plastic adjustment to local conditions (fig 2) However it is often possible to demonstrate a course of the fibrils in which at least a close relationship to plasma extensions from individual reticular cells cannot be overlooked This observation of the relationship of fibrils to plasma, which is not imitated by spatial superimposition, may contribute to the discussion on the actual place of formation of the filaments. Observations that the precursors of collagen fibrils are expelled from the fibroblasts in the form of unarranged packs (11) may be of importance in this connection. They tend to support the hypothesis that the site of fibrillogenesis is in the ectoplasma, which separates out as structurally still not differentiated protofibrils (10)

Therefore the reticular fibre is not a transitory phase in the development of the typical collagen fibre in a fibrocytic milieu. The argyrophil fibre in fibrebundle form remains a special structural feature of the reticular cell tissue, which characterises also its morphologically observable manifestations.

The *follicles* of spleen and lymphatic tissue are a characteristic, but in their structure only little differentiated part of the



Fig 1 Reticular cells with mitochondria, vesiculation, lipoids and continuous outlines (Mesent. lymphnode, monkey 7900× 3)

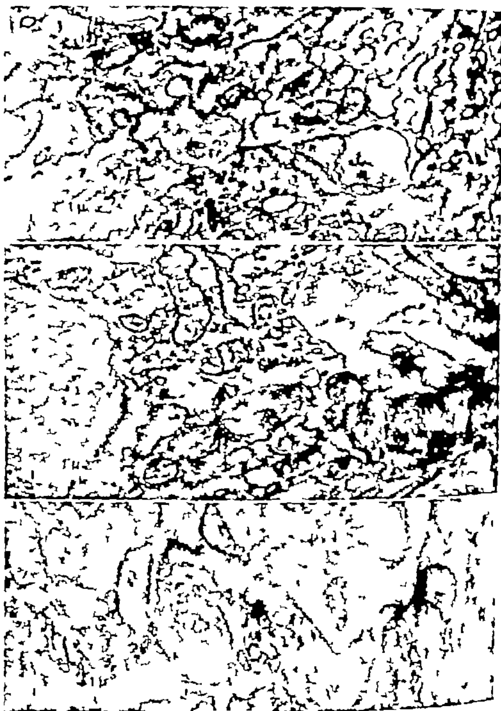


Fig I tercellular-clefts with cross-sectioned fibrils (Mesent lymphnode monkey) 7900 4 b 7900 4 c 7900 6)

reticular cell component. They consist of cells which are rich in plasma and in varying degree of approximation and usually form a distinct net without formation of argyrophil fibres. The active, loosened follicle, which shows plenty of mitoses, consists of large macrophagocytic elements. The same conditions and changes are found in the follicles of the lymph nodes.

The *lining cells of the lymph paths* in the lymph nodes are also connected with each other and with the surrounding reticular cells by lattice fibres and, therefore, may be considered to be endothelially positioned reticular cells according to flow conditions. Because of these findings, which also apply to sections of a similar structure in the blood vessels of the reticulum in spleen and bone marrow these parts of the lymph and blood vessels of the reticular cell tissue are termed *sinusoids*. The loose structure of their walls differs markedly from the endothelium of ordinary blood and lymph vessels bordered by a basal membrane. The stellate cells of the liver form the cellular component of a lattice fibre tube and, therefore, show the structural characteristics of sinusoids. Their function and appearance correspond to those of the reticular cells.

By the *electronmicroscope* it is possible to study the true nature of the cells bordering the sinusoids, where the reticular cells arrange to coating cells without basal membrane. Submicroscopically continuous clefts show between both the extensions of the regularly flat forms and the bulging activated cells. They begin at the surface of the sinusoid as a pore, occasionally slightly funnel-shaped, and are continuous with similar clefts between adjacent reticular cells (4). As it has been possible to see intercellular clefts between the border cells of the rat's spleen (11) between the overlapping Kupffer cells in the liver of mouse (6) and rat (7) the sinusoids lined with retothelial elements may be assumed to share a common structural principle. The same structure is found in the sinusoids of spleen and liver in man (2, 9) In large surfaces this common structural principle may explain the ease and rapidity with which storage and phagocytosis occur and, because of the absence of an obturating basal membrane, would also be of special significance in explaining permeability in the region of the sinusoids.

The endothelial cells of a typical capillary wall neither possess nor assume the function or form of a reticulogenic border cell. The principal differences between ordinary endothelial cell and reticulogenic border cell in both function and structure cannot be overlooked.

The *histiocytes* (5) have a function similar to that of the primary fixed reticular cells and the mobile reticulogenic border cells of the sinusoids. With their fibrillary texture, typical of that seen in the joining together of reticular cells, they clearly show their common origin with the reticular cell (1) Through these histiocytes, developing in the subendothelial and adventitial layer of the vessels, the system of reticular cell tissue becomes practically ubiquitous (3).

The reticular cell tissue forms with its net of argyrophil fibres the stroma of lympho-reticular tissue and bone marrow and the structural element of the spleen. Reticular coating cells are the endotheloid elements lining the sinusoids of the bone marrow spleen, liver and lymph nodes the essential feature distinguishing them from the general vascular endothelium is the absence of a basal border by a collagen basement membrane. The extended portion of the system is formed by mobile histiocytes in the undifferentiated circumvasal and subendothelial zones, where they can also be formed. Submicroscopically the reticular tissue, including the lining cells, show cell borders and continuous intercellular clefts which, owing to the spatial connection of the cells, communicates with the sinusoids. Cross striated fibrils are seen in the amorphous ground substance of the widened clefts as oriented bundles they correspond to the histological argyrophil reticular fibre. These fibrils occur not only extracellularly but sometimes are connected with the ectoplasm of the reticular cells, which has to be considered for the methode and place of fibrillogenesis. The close histological and functional resemblance of reticular cells, lining cells and histiocytes established the existence of this partly preexistent, partly developed tissue. By prooved exclusion of the ordinary endothelium of blood and lymph-vessels, it represents the substrat of the retothelial system.

Summary

The reticular cell tissue forms with its net of argyrophil fibres the stroma of lympho-reticular tissue and bone marrow and the structural element of the spleen. Reticular coating cells are the endotheloid elements lining the sinusoids of the bone marrow spleen, liver and lymph nodes; the essential feature distinguishing them from the general vascular endothelium is the absence of collagen basement membrane. Submicroscopically the reticular cell tissue, including the lining cells, show cell borders and continuous intercellular clefts which, owing to the spatial connections of the cells, communicate with the sinusoids. Cross striated fibrils are seen in the amorphous ground-substance of the widened clefts as oriented bundles they correspond to the histological argyrophil reticular fibre. These fibrils occur not only extracellularly but sometimes are connected with the ectoplasm of the reticular cells, which has to be considered for the mode and place of fibrillogenesis.

Résumé

Le tissu réticulaire forme avec son filet de fibres argentaffines le stroma du tissu lympho-réticulaire et de la moelle osseuse, ainsi que l'élément constitutif de la rate. Les cellules bordant les sinusoïdes de la moelle osseuse, de la rate, du foie et des ganglions lymphatiques sont des éléments réticulés. Ils diffèrent de façon essentielle des autres endothelia par le manque d'une membrane collagène basale. Au niveau submicroscopique le tissu réticulaire, y compris les cellules pariétales, montre des bords cellulaires et des fentes intercellulaires continues qui grâce aux interstices qui se trouvent entre ces cellules communiquent avec les sinusoïdes. Dans la substance fondamentale des fentes élargies se trouvent des fibrilles striées. Elles y forment des faisceaux qui correspondent histologiquement aux fibres réticulaires argentaffines. Ces fibrilles ne se trouvent non seulement en dehors des cellules mais sont aussi parfois en contact avec l'ectoplasme des cellules réticulées. Cette observation [illegible] une certaine importance pour le mode et la localisation de la formation des fibrilles.

Zusammenfassung

Das retikuläre Zellgewebe bildet mit seinem Netz argyrophiler Fasern das Stroma im lympho-retikulären Gewebe und Knochenmark, sowie das Bauelement von Milz. Wandständige retikuläre Zellen sind die endothelähnlichen Elemente der Sinusoide im Knochenmark, Milz, Leber und Lymphknoten. Sie unterscheiden sich grundsätzlich von den übrigen Gefäßendothelien durch das Fehlen einer kollagenen Basalmembran. Submikroskopisch zeigt das retikuläre Gewebe, einschließlich der Wandzellen, Zellgrenzen und kontinuierliche interzelluläre Spalten, die mit den Sinusoiden kommunizieren. In der amorphen Grundsubstanz erweiterter Spalten finden sich quergestreifte Fibrillen, die als gerichtete Bündel den histologisch erkennbaren argyrophilen retikulären Fasern entsprechen. Diese Fibrillen finden sich nicht nur extrazellulär sondern stehen bisweilen mit dem Ektoplasma der retikulären Zellen in Verbindung, was für Art und Lokalisation der Fibrillenbildung von Bedeutung ist.

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Acta haemat. 32: 200–205 (1964).

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Investigations on the Electrophoretic Mobility of Factor X on Starch Block

By C. Gardikas, G. Arapakis, J. Jordanoglou and D. Thomopoulos

Factor X is required for the production of both intrinsic and extrinsic prothrombin activator. Rabiner and Kretchmer (10) studied a boy with a marked deficiency of the factor in both phases whose father had a marked deficiency in the first phase and a moderate deficiency in the second, whereas his sister had a moderate deficiency in the first phase only. Furthermore, they observed an apparently normal person in whom the activity of factor X estimated by Quick's one-stage prothrombin test was 87 % and by the thromboplastin generation test it was 32 % of the normal. Gardikas et al. (5) assessed separately the two activities of the factor X on 50 Dindevan treated patients, and found that 6 of them presented a marked discrepancy between the defects in the two phases. In addition, amongst 100 persons apparently normal the same workers found 4 with a marked reduction in activity in the intrinsic phase only and two with a similar reduction in activity in the extrinsic phase. These observations suggest that the two activities of the factor may vary independantly.

In the present paper fractions of human serum isolated by starchblock electrophoresis have been investigated in reference to their ability to correct the deficiency in either activity of factor X.

Material and Methods

Plasma and serum was obtained from (a) normal persons, (b) 3 Dindevan-treated patients presenting a marked reduction in both activities of factor X, (c) 3 Dindevan-treated patients with a marked reduction in the activity in the intrinsic phase only and (d) 3 Dindevan-treated patients with a marked reduction in activity in the extrinsic phase only. All patients treated with Dindevan were receiving the drug for 8 days or more.

Plasma: The venous blood was collected with siliconed syringes, mixed immediately with 1/10 volume of 3.8 % trisodium citrate in glass tubes and then centrifuged at 3000 rev/min for 15 minutes. The supernatant plasma was pipetted off and used either immediately or stored at —20 °C.

Serum: Venous blood was incubated at 37 C for 4 hours, and at 4 °C for further period of 16 hours. Serum was then separated by centrifugation at 3000 rev/min for 15 minutes.

Veronal buffer pH 8.6, ionic strength 0.05 was used for preparing the starch block and for the buffer compartments of the electrophoresis tank. Composition sodium diethyl barbiturate 10.3 g, diethyl barbituric acid 1.84 g, made up to 1 litre with distilled water

Glyoxaline (imidazole) buffer pH 7.3, prepared as described by BIGGS AND MACFARLANE (3) was used as diluent and for elution.

Assay of the activities of factor X. 1) For the *extrinsic phase* the method of BACHMAN et al. (1) was used except that charcoal-filtered instead of asbestos-filtered ox plasma was used as substrate according to DENSON' modification (4). 2) For the *intrinsic phase* the method described by GARDIKAS et al. (5) based on the thromboplastin screening test of HICKS AND PITNEY (7) was used. Details of the techniques of both assays are given in the section on the assessment of the corrective activity of the eluates.

Starch-block electrophoresis. Potato starch (500 g) was mixed with 400 ml buffer solution to form thick paste. The paste was then poured into mould (29.5 × 6.5 × 1.5 cm.) made of heavy wax paper resting on a long glass plate supported at the sides by wooden blocks. In detail, the technique as described by KUNKEL (9) was followed. The slit was made through the entire thickness and across the middle of the block to within one cm of the edges. The block was placed on the horizontal plate of the electrophoresis apparatus E. C. 401, Research Model. Filter paper wicks (Whatman 3 MM) connected the block to the buffer tanks. A voltage of 400 V and current of approximately 15 mA was applied. Electrophoresis was carried out for 14 hours at 2 –4 °C. At the end the starch block was divided transversely into 1 cm wide segments. A yellow band due to bilirubin marked the position of the albumin band. Each starch segment was placed in narrow-stemmed ground glass funnel, and the retained material was eluted with 5 ml of mixture of equal volumes of glyoxaline buffer and isotonic saline. The fractions of albumin and γ-globulin were discarded, as preliminary experiments showed that they possess no factor activity The eluates from the α_1- α_2- and β-globulin fractions were then concentrated to one-tenth of the original volume by dialysis for 12 hours against Carbowax at 4 °C. The protein concentration in the eluates was determined by the biuret method (6). The protein concentration of the eluates was then brought to the same level by adding glyoxaline buffer-NaCl mixture.

Assessment of the corrective ability of the eluates: 1) For the *extrinsic phase* mixtures were prepared containing 0.1 ml of each of the following reagents a) plasma to be tested, b) charcoal-filtered ox plasma,) "Stypven -ovolecithin mixture. T 10 ml of Stypven solution 1 100000, 0.2 ml of 10% alcoholic solution of ovolecithin (B. D. H.) was added, d) glyoxaline buffer or one of each of the eluates of α_1- α_2- and β-globulin fractions of the serum and) M/40 $CaCl_2$. The clotting times were compared with those of calibration curv obtained by performing the test on series of mixtures containing serial dilutions of pooled normal citrated plasma (1 10, 1 20, 1 40, 1 80, 1 160, 1 320, and 1 640) corresponding to factor X levels of 100 to 1.6% of the normal.

For the *intrinsic phase* mixtures were prepared containing 0.1 ml of each of the following reagents a) plasma to be tested diluted 1 10 with the glyoxaline buffer-isotonic saline mixture, b) charcoal-filtered ox plasma diluted 1 10 with the same buffer

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Powdered wood charcoal, obtainable from Messrs. Griffiths and George Ltd., Alperton, Middlesex, England.

NaCl mixture,) brain cephalin" (2) d) glyoxaline buffer or one of each of the eluates of α_1 α_2- and β-globulin fractions of the serum and) M/40 $CaCl_2$. The clotting times of the substrate plasma were determined 5 and 6 minutes from the beginning of the incubation, and compared with those of a calibration curve obtained by performing the test on a series of mixtures containing serial dilutions of pooled normal citrated plasma.

Results

1 Dindevan-treated patients with both activities of factor X equally reduced. As table I shows, the reduced activity of factor X in the intrinsic phase was corrected by any of the eluates of the α_1 α_2- or β-globulin fractions of normal serum. The reduced activity of factor X in the extrinsic phase was markedly corrected by the α_1 and the α_2 globulin fraction eluates of the β-globulin fraction had poor corrective activity

Table I

Corrective ability of α α_2- and β-globulin fractions of normal serum on the reduced activities of factor X of plasma of Dindevan-treated patients. Each figure represents the mean of four determinations.

Dindevan-treated patients	Factor X activity of plasma (% of normal)							
	In the intrinsic phase after adding of				In the extrinsic phase after adding of			
		globulin of normal serum				globulin of normal serum		
	buffer	α_1	α_2	β	buffer	α_1	α_2	β
1	18	60	60	60	10	55	60	25
2	22	0	65	63	11	60	62	20
3	20	70	65	62	10	60	65	[illegible]
4	10	63	62	68	15	55	48	18
5	12	55	65	65	20	57	65	35

2 Dindevan-treated patients with a reduction in activity in the intrinsic phase only of factor X Three persons of this group were investigated. As table II shows, the reduced activity in the intrinsic phase was corrected by any of the eluates of α_1 α_2 or β-globulin fractions of normal serum. Eluates of α_1 α_2 and β-globulin fractions of serum of Dindevan treated patients with reduced activity in the extrinsic phase only showed similar ability Fractions of serum of Dindevan treated patients with a reduction in both activities of factor X had no corrective effect.

3. Dindevan-treated patients with a reduction in activity in the extrinsic phase only of factor X Three persons of this group were investigated. Eluates from the α_1 and α_2 globulin fractions of normal serum exhibited marked corrective activity whilst eluates from the β-globulin fraction brought about a less marked correction. Significant corrective ability was also exhibited by the α_1 and

Table II

Corrective ability of α_1-, α_2- and β-globulin fractions of various sera on plasma of Dindevan-treated patients with reduction only in the intrinsic activity of factor X. Group I: Dindevan-treated patients with reduction in both activities of factor X. Group II: Dindevan-treated patients with reduction in the extrinsic activity of factor X only

Each figure represents the mean of four determinations.

Dindevan-treated patients	Factor X activity of plasma in the intrinsic phase (% of normal) after adding of									
	buffer	globulin of normal serum			globulin of patients of Group I			globulin of patients of Group II		
		α_1	α_2	β	α_1	α_2	β	α_1	α_2	β
1	15	60	70	55	12	15	15	65	65	65
2	20	65	60	65	12	22	15	60	68	62
3	15	68	55	68	15	15	22	75	70	70

α_2-globulin fractions of serum of Dindevan treated patients with reduced activity in the intrinsic phase only. Similar fractions of serum of Dindevan-treated patients with a reduction of both activities of factor X exhibited no corrective ability.

Discussion

The electrophoretic mobility of factor X has been studied by various workers. Schultze and Schwick (11), Higashi et al. (8), Tsevrenis et al. (12) and Denson (4) found that factor X migrates with the α-globulins. The present investigations on the electrophoretic mobility of factor X differ from the previous ones in two respects. (a) In all previous experiments filter paper electrophoresis was used. Starchblock, however, presents some advantages over filter paper electrophoresis. According to Kunkel (9) there is much absorption on filter paper and relatively little on starch grains. In addition, starch block has the advantage that much more serum can be applied and more material can be obtained for the assessment of corrective activity. (b) In all previous experiments the activity of the various fractions of normal serum was assessed by the correction of the onestage Quick's test time. Since the findings of Rabiner and Kretchmer (10) and Gardikas et al. (5) suggest that the two activities of the factor may vary independently the method of separate assessment of the two activities of the factor used in the present experiments would be considered more satisfactory.

The findings of the present investigations that the activity of factor X in the intrinsic phase is located in the α, α_2- and β-globulin

fractions of serum, whereas the activity in the extrinsic phase is located mostly in the α_1 and α_2-globulin fractions are in agreement with those of RABINER AND KRETCHMER (10). Studying a patient with typical factor X deficiency these workers found that eluates of α_1-, α_2- and β-globulin fractions of normal serum obtained by starch-block electrophoresis corrected markedly the thromboplastin generation whilst eluates of β-globulin fraction had poor corrective ability on the prothrombin activity on the same patient.

Acknowledgements: The receipt of a grant from the Royal Hellenic Research Foundation is gratefully acknowledged; thanks are due to Miss Electra Ioannidou for technical assistance.

Summary

Eluates of α_1-, α_2- and β-globulin fractions of normal serum obtained by starch block electrophoresis markedly corrected the reduced activity of the factor X in the intrinsic phase, whereas the reduced activity of the factor in the extrinsic phase was corrected mostly by α_1- and α_2-globulin fractions. The corrective ability of the various fractions of normal serum was assessed on plasma of Dindevan-treated patients who exhibited a deficiency of factor X in both activities as well as on similarly treated patients with a deficiency in either of the phases only.

Résumé

Des éluats de fractions des α_1-, α_2- et β-globulines de sérum normal obtenus par électrophorèse sur bloc d'amidon corrigent considérablement l'activité réduite du facteur X dans sa phase intrinsèque, tandisque l'activité réduite du facteur dans sa phase extrinsèque est corrigée surtout par les fractions des α_1- et α_2-globulines. L'activité correctrice des fractions du sérum normal est déterminée sur du plasma de patients recevant du Dindevan avec une réduction du facteur dans les deux phases, ainsi que dans l'une des deux.

Zusammenfassung

Die durch Stärkeblock Elektrophorese erhaltenen Eluate von α_1-, α_2- und β-Globulinfraktionen von normalem Serum korrigierten die verminderte Aktivität von Faktor X bedeutend in der Intrinsic-Phase, während eine verminderte Aktivität in der Extrinsic-Phase hauptsächlich von α_1- und α_2-Globulinfraktionen korrigiert wurde. Die Korrektionsfähigkeit der verschiedenen Fraktionen von normalem Serum wurde an Plasma von mit Dindevan behandelten Patienten bestimmt, die einen Mangel an Faktor X für beide Phasen oder nur für eine der beiden Phasen aufwiesen.

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Anticoagulant antiplaquettaire passager et cellules de Hargraves au cours d'une ostéomyélosclérose

Par G. Mayer, S. Mayer et R. Waitz

L'observation rapportée ici est celle d'une ostéomyélosclérose polyglobulique. Il s'y rencontre un syndrome hémorragique modéré, dû à une atteinte vasculaire peu à peu renforcée par une thrombopénie progressive. C'est là un tableau assez souvent rencontré en milieu hématologique. Ce qui est particulier à notre cas est la brève survenue d'un inhibiteur dirigé contre l'action des plaquettes dans l'hémostase plasmatique. En même temps baisse la prothrombine et ses accélérateurs, proconvertine (F VII) et proaccélérine (F V) ont un comportement anormal.

Observation

Humbert Jacque Z... Solange, née le 18 avril 1910, se plaint en 1934 d'une gêne épigastrique due à une splénomégalie. Celle-ci s'accroît au cours des années suivantes. En 1958 apparaît l'érythrose, en 1959 est constatée la polyglobulie. En décembre 1960 surviennent des ecchymoses, du purpura des membres inférieurs et aussi de petites épistaxis à l'occasion d'un rhume.

En mars 1961 la rate énorme (42 × 25 cm) plonge dans la fosse iliaque gauche occasionnant refroidissement, cyanose et paresthésies des extrémités inférieures. Sur les radiographies du crâne, du bassin, des fémurs, du tibia se dessinent des zones très étendues et presque éburnées d'ostéosclérose. Le 15 mars, une trépanoponction sternale est suivie d'un hématome assez large et le suintement sanguinolent de la plaie persiste une quinzaine de jours. La compression du bassin en vue de la radiographie le 17 mars détermine des ecchymoses de la hanche et des genoux. Le 16 avril, la malade reçoit une injection intra-veineuse de 200 microcuries de radiophosphate de chrome et du 17 au 21 de la vitamine K 1 par voie parentérale. Le 23 mai une ponction-biopsie splénique est pratiquée sans incident.

Pourtant les exacerbations du syndrome hémorragique se succèdent, en janvier-mars 1962 où la rupture d'une saphène interne produit un gros hématome et en juin-août. L'état général, malgré un certain amaigrissement, reste satisfaisant. Mais en juillet 1962 apparaissent des ganglions sous-maxillaires et inguinaux, gros comme une noisette



Fig 1 Trépanoponction sternale 14 mars 1961 H. E. S. Moelle osseuse Sclérose dense avec nombreuses cellules normoblastes, granulocytes à différents stades, cellules réticulaires, mégacaryocytes dont l'un fait saillie dans un sinus dilaté

dues et mobiles. Il est certain que tant en ce qui concerne le volume de la rate que l'intensité des troubles périphériques, la densification osseuse, la fréquence et l'étendue du purpura, des ecchymoses et des hématomes, on assiste à une détérioration de la situation, lente mais accélérant peu à peu. Après le 2 avril 1963 la malade est perdue de vue.

L'évolution hématologique connaît deux périodes: 1 De mars à novembre 1961 la polyglobulie est stable et normochrome, entre 5,19 et 6,18 millions d'hématies par mm³ dont 13 à 29‰ de réticulocytes, avec 106 à 112% d'hémoglobine Sur les 19 300 leucocytes par mm³ 17,5‰ sont des granulocytes immatures qu accompagnent 4 normoblastes pour 100 globules blancs (aleurs moyennes) 2 En février–mars 1962, au moment d'une recrudescence des hémorragies, les hématies tombent à 4 71 millions par mm³ et l'hémoglobine à 90% En même temps la réticulocytose atteint 102‰. La preuve d une spoliation sanguine correspondante ou d une hémolyse pu être faite. Dès ce moment la leucocytose augmente. Lentement la polyglobulie se rééstalle elle tteint 5,65 millions par mm³ que le 1er avril 1963. Mais l'hémoglobine baisse et est alors plus qu à 80‰, d'où forte hypochromie. La réticulocytose se maintient entre 20 et 24‰. La leucocytose gravite autour de 37 000 cellules par mm³ dont 20‰ de granulocytes immatures et le taux des normoblastes est maintenant de 10,5 pour 100 leucocytes (valeurs moyennes).

La recherche de cellules de Hargraves ne trouv le 23 mai 1962 que deux tart-cells sur 3000 polynucléaires mais le 1er août deux *cellules typiques de Hargraves* et 18 tart-cells sont vues sur .000 polynucléaires, à plusieurs contrôles.

Etude morphologique: La trépanoponction sternale (14 mars 1961) montre une *myélosclérose* très dense, renfermant de grands sinus vasculaires et très riche en cellules hématopoïétiques. Elle contient de petites travées ostéoïdes. Des apositions de même nature, parfois bourgeonnantes, se plaquent sur l'os ancien (fig. 1). La ponction splénique met en évidence une *sclérose myéloïde de la rate*.

Etude de l'Hémostase

Matériel et Méthodes

1. Prélèvement du sang et préparation des fractions se font selon les procédés habituels. La céphaline est obtenue à partir d'une thromboplastine de cerveau humain (BELL et ALTON).

2. Les *méthodes courantes* utilisées sont: le signe du lacet (brassard sphygmomanométrique), le signe de la ventouse (angiostéromètre de LAVOLA: 30 cm Hg × 60 sec.), la numération des plaquettes (FEISSLY et LUDIN), le temps de saignement (DUKE), le temps de coagulation du sang complet (LEE WHITE), le temps de recalcification du plasma (HOWELL), le temps de prothrombine (QUICK), les temps de proaccélérine, de proconvertine-facteur X, de prothrombine vraie (OWREN-KOLLER), la consommation de la prothrombine (par temps de QUICK du sérum réapprovisionné en fibrinogène, après 4 heures × 37°), le temps de thrombine (0,1 ml de plasma + 0,1 ml d'une solution à 2 U.I. de thrombine). Le taux du fibrinogène (LAROUX), le temps de lyse des euglobulines (SUMMARY et coll.), le dosage du facteur X (KOLLER), le dosage du facteur antihéparinique des plaquettes F.P. 4 (DEUTSCH), le test de la génération de la thromboplastine de BIGGS et DOUGLAS (TGT), le dosage de la globuline antihémophilique (F VIII) et du facteur Christmas (F IX) au moyen du TGT. Dans ce test, où chaque épreuve comporte une succession de temps de coagulation, et dans tous ceux qui en dérivent, seul le temps de coagulation minimum (TCm) est retenu. Les facteurs sont exprimés en pourcent de la normale (N) selon la fraction analysée.

3. Des méthodes plus particulières nous ont servi pour la *détection de l'inhibiteur*.

a) *Céphaline préincubée et réutilisée dans le* TGT. La céphaline est diluée 1:10 dans la fraction à tester et comparativement dans une fraction normale, puis préincubée à 37 °C. Au moment choisi un échantillon est prélevé, rédilué 1:10 en saline (concentration finale de la céphaline 1:100) et utilisé comme réactif plaquettaire dans un TGT normal.

b) *Céphaline ou plaquettes préincubées et réutilisées dans le temps de thromboplastine partielle* (TTP): 0,2 ml de céphaline 1:100 en saline ou d'une suspension décongelée de plaquettes normales (210 000–290 000/mm³) sont préincubées à 37 °C en présence de 0,1 ml du plasma à tester et comparativement de plasma normal. Au moment voulu, 0,1 ml de plasma normal est ajouté à ce mélange qui fournit à deux mesures du temps de céphaline ou de thromboplastine partielle (TTP).

c) *Incorporation de la céphaline par une mixture incomplète en présence de plasma*. Plasma adsorbé sur sulfate de baryum (plasma Ba) et sérum pris sur thromboplastine normaux et de préférence non dilués, sont mélangés, recalcifiés et mis à incuber à 37 °C comme dans le TGT. C'est ce que l'on peut appeler mixture incomplète. Le substrat est constitué par le plasma à examiner et comparativement du plasma normal, déplaquetté et en tubes de 0,1 ml. Aux intervalles voulus, 0,1 ml de mixture est:

1° variante, soit soufflée avec 0,1 ml de $CaCl_2$ M/40 dans le substrat où pré-incube depuis 30 secondes, 0,1 ml de céphaline 1:100

– 2° variante, soit reportée, 30 secondes avant son échéance, dans un tube contenant 0,1 ml de céphaline 1:100. Après 30 secondes le tout est repris et soufflé avec 0,1 ml de $CaCl_2$ M/40 dans le subst

Examen	N	7.3. 61	8.3.	21.3. × 23.3.	7.4.	21.4	17.5.	27.6.	24.10. × 26.10.	16.2. 62	23.3.	2.8.	1.4. 63
Plaquettes 10^3/mm³	150,0–350,0	116,8	50,2	148,0	125,6	75,2	81,8	96,0	107,2	51,0	59,8	100,8	61,0
Temps de saignement, min., sec.	≤3		–	1,30	4	4,30	2	4,30	4	3		3,30	5,30
Lacet, ventouse	0			0	(+)	0	0	0	(+)	0		0	+
Retraction	+/++	–	–	++	+	++	++	++	+	+	–	+	+
Ma mm	≥30							–	× 62	52		–	–
FP 4 u. hepar neutr p. 10^9 plaq	0,024 0,004			× 0,021		–				0,026		0,024	
Temps de coagulation, min., sec	6–11		–	16,30	11	9	10	17 45	14,30	13,15		11	11 45
Temps de recalcification, sec.	témoin entre ()			205 (290)	206 (186)	213 (189)	242 (151)	165 (144)	204 (163)	129 (137)	–	102 (144)	147 (139)
Prothrombine du sérum, %	2–9			16	13	26	23	11	6	13	–	6	10
Prothrombine du plasma, %	100			80	79	75	41	80	68	73	–	68	97
F VII F X (plasma)	100			100	61	58	31	96	78	83		83	98
Prothrombine vraie (F II)	100			100	79	67	58	106	82	84		79	93
F V %	100			88	94	85	90	83	97	98		100	100
F X, %	100			× 70 (plasma)	85 (sérum)	88 (sérum)	90 (sérum)		85 (plasma)	85 (plasma)			
F VIII	100			× 108	65					N			–
F IX, %	100			× 116	100					N			–
Fibrinogène mg/	350–550			389	408		332		351	533		390	354
TGT plaquettes	N			× N	N	N	DLNC	N	N	DL			
plasma Ba	N			× N	DLNC	DMNC	N	N	N	DLNC			
sérum	N			× N	N	DMNC	DM	N	N	N	–		–
plasma Ba + sérum	N			N	DMNC	DMNC	DM	DL	DM	DL			–
Inhibiteur	0			× 2+	3+	3+	1+	0	0	0	–	–	

N = normal L = léger NC = normalisé par congélation
D = déficit M = modéré

Temps de lyse des euglobulines (N ≥3 heures) >3 heures (2 ril 1963)

Dans la première variante, la réaction entre la mixture incomplète et la céphaline se fait dans le plasma-substrat dans la seconde elle a lieu hors de ce dernier

Pour les applications spéciales de ces différentes méthodes, courantes ou particulières, on se reportera aux tableaux.

Résultats

Les dernières étapes de l hémostase sont normales. Le fibrinogène varie entre 333 et 408 mg %. Le temps de thrombine concorde avec celui des témoins. Il n y a pas de lyse exagérée du caillot. Par contre, partout ailleurs les troubles sont sporadiques ou constants (tableau I)

1 Le taux des *plaquettes* de mars à juin 1961 oscille entre des valeurs faibles et subnormales (valeur moyenne 102.700/mm³). De juillet à octobre il est normal (162.800/mm³) A partir de février 1962 il reste bas (68.700/mm³) quoique très variable. Cependant l activité dans le TGT (FP 3) est presque toujours intacte. L activité antihéparinique (FP 4) ne sort pas des limites habituelles. L amplitude Ma du thrombélastogramme est convenable et la rétraction du caillot bonne, même en période thrombopénique.

2. La *fragilité vasculaire* et le médiocre ***allongement du temps de saignement*** sont inconstants d un examen à l autre mais réapparaissent sans cesse. D abord indépendants de la thrombopénie ils finissent par s en trouver majorés.

3 *L hypocoagulabilité* est discrète mais quasi constante. D'avril à juin 1961 la ***mauvaise consommation de la prothrombine*** est indépendante des poussées de déplaquettisation quoique celles-ci l accentuent. Plus tard elle suit la thrombopénie. Or le TGT ne révèle qu un comportement normal des fractions ou le plus souvent, un médiocre déficit du type facteur contact. Les facteurs VIII, IX et X atteignent des valeurs satisfaisantes.

4 Ces discordances amènent sur la piste d un ***inhibiteur*** manifeste au cours de la formation du procoagulant («thromboplastine») endogène. Ainsi le 23 mars, plasma Ba et sérum de la malade aux dilutions ordinaires agissent normalement dans le TGT (TCm 10,5/témoin 10 0 sec) Par contre le mélange des fractions non diluées est déficitaire (15 8/8,2 sec) et somme toute se comporte comme une mixture incomplète sans céphaline. Mais la mixture incomplète sans céphaline pour malade et témoin présente une activité comparable (15 8/17,5 sec)

5. Cette *activité dirigée contre la céphaline ou les plaquettes* s affirme de diverses manières

Tableau II

Neutralisation de la céphaline préincubée et réutilisée dans le TCT (matériel et méthodes 3)
TCm en sec. Valeurs normales entre ().

Fraction préincubée avec la céphaline	date	23 mars 1961		7 avril 1961		21 avril 1961		17 mai 1961	
	congélation	6 et 12 jours		4 jours		4 jours		2 jours	
	37 ×	0	60 min	0	60 min	0	60 min	0	60 min
Plasma		12,0 (11 7)	16,7 (11 4)	10,0 (8,3)	9,9 (9,0)	10,9 (8,5)	10,4 (10,7)	10,8 (9,2)	9,8 (8,7)
Plasma Ba		20,3 (15,8)	16,4 (13,6)	12,4 (8,9)	12,0 (10,2)	12,1 (10,9)	18,8 (13,5)	10,7 (9 4)	11,8 (10,6)
Sérum		8,9 (8,3)	8,5 (9,9)	10,0 (8,4)	9,6 (8,4)	9,9 (8,0)	11,9 (8,5)	11,8 (11,8)	12 1 (11,2)
Sérum Ba		19,0 (10,5)	20,2 (10,3)	32,0 (14,9)	55,5 (17,5)	–		–	–
Sérum 56 × 30 min		19 4 (11,8)	20,9 (13,9)						–
Sérum / Plasma									

Tableau III

Neutralisation de la céphaline ou des plaquettes préincubées et réutilisées dans le TTP (matériel et méthodes 3 b)
TC en sec. Valeurs normales entre ()

Matériel préincubé	date	23 mars 1961		17 mai 1961		27 juin 1961		16 février 1962	
	congélation	5 jours		2 jours		0		3 jours	
	37 min	0	60	0	60	0	60	0	60
Plaquettes		119 4 (89,6)	142,2 (139,3)	129 7 (92,1)	116,6 (70,8)	73,5 (74 1)	74 1 (66,0)	71,0 (66,5)	85,6 (85,2)
Céphaline		136,3 (117,0)	179,0 (159,4)	156,3 (119,8)	160,6 (126,0)	115,8 (105,7)	99,5 (87 1)	75,2 (68,9)	86,7 (86,3)

Tableau II

Effet inhibiteur du substrat plasma malade ec céphaline préincubée
(cf. matériel et méthodes 3) TCm en sec. Valeurs sur substrat plasma normal entre ()

Variante	date congélation	7 vril 1961 26 jours	21 vril 1961 11 jours 28 jours	17 mai 1961 1 jour	27 juin 1961 frais	24 octobre 1961 frais
Céphaline 30 sec. dans substrat		22,8 (11 7)	20,6 (11 4) 20,3 (11,3)	18,4 (17 1) 18,5 (15,5)	12 0 (11,9)	9 7 (11,0)
Céphaline 30 sec. dans mixture		11,9 (12,8)	10,1 (12,0)	9,5 (10,0)	9,9 (9,5)	–

Mixture incomplète: plasma IIa 1 3; sérum 1:10
Substrat malade ec 20% substrat normal.

Plasma du 7 vril 1961 Mixture incomplète normale: plasma IIa 1:5, sérum 1:10. Céphaline dans substrat malade

A

Effet de la dilution du substrat

Dilution en saline	T Cm sec.
non dilué	22,8 (11 7)
1 2	26,1 (19 7)
1 5	28,1 (27,0)

B

Effet du taux de céphaline

T ux de céphaline	T Cm sec.
1 : 100	22,8 (11 7)
1 : 50	10,1
1 10	15,3

a) *La céphaline réutilisée dans le* TGT est plus détériorée au contact des fractions malades que des normales (tableau II). Néanmoins, dans ces conditions un effet inhibiteur provient déjà du sang normal ; de plus il varie d'une expérience à l'autre.

b) *La céphaline ou les plaquettes normales reprises dans le temps de thromboplastine partielle* (TTP : temps de céphaline) allongent ce temps lorsqu'elle ont été mélangées d'abord à du plasma malade (tableau III). Il est vrai qu'ici du plasma malade se trouve ajouté au plasma normal au moment de la recalcification et pourrait exercer son action anticoagulante ailleurs que sur la céphaline ou les plaquettes.

c) *Le pouvoir procoagulant d'une mixture incomplète normale* est plus faible sur plasma malade que sur plasma normal, lorsque ceux-ci contiennent déjà la céphaline. Mais que la mixture reçoive la céphaline directement, peu avant d'être testée, son activité apparaît identique sur les deux substrats (tableau IV). L'incorporation de la céphaline dans la mixture normale est gênée en présence de plasma malade. Cet effet inhibiteur est *supprimé par dilution* du substrat malade (tableau IV A). Il est *atténué par un excès de céphaline* (tableau IV B).

La mixture incomplète *malade* ne manifeste aucun pouvoir anticoagulant sur plasma normal lorsqu'elle est d'abord mise en contact directement avec la céphaline (2e variante). Mais il n'en est plus de même si des plaquettes sont substituées à la céphaline. Lorsque la mixture est transportée sur le substrat contenant déjà la céphaline (1re variante) l'effet inhibiteur se manifeste souvent (tableau V). Ces faits pourraient s'expliquer par l'absence dans la mixture elle-même d'un *cofacteur* de l'inhibiteur apporté par les plaquettes et surtout par le substrat, peut-être la prothrombine comme dans l'observation de Loeliger (9).

L'activité inhibitrice *se limite à la céphaline et aux plaquettes*. Elle ne s'exerce ni contre la prothrombine, ni contre les facteurs VII, X, VIII ou IX (tableau VI). Dans tous les essais, elle a presque toujours un *caractère immédiat* que n'accroît pas l'incubation à 37 °C, même pendant une heure. Elle est *passagère* : atteint son maximum le 7 avril 1961, décline après le 21 et s'éteint entre le 17 mai et le 27 juin, ayant duré 3 mois environ.

Elle se retrouve dans le *sérum*, n'est *pas adsorbable* sur sulfate de baryum mais, *thermolabile*, supporte mal 56 °C pendant 30 minutes (tableau VII A, voir aussi tableau II). Elle *disparaît à —30 °C* entre 27 et 28 jours, lorsqu'elle est au plus fort, à 9 jours déjà quand elle se trouve sur son déclin, quoiqu'à ce moment le plasma laissé

Tableau VIII

Labilité de la proaccélérine (F V) à 37 °C et à —30 °C.

Date	23 mars 1961	7 avril 1961	21 avril 1961	17 mai 1961
30 °C. ×	4 jours	7 jours	3 jours	4 jours	1 jour

1 Conservation à la congélation.

valeurs en pourcents de la normale, entre () taux de F V dans le plasma oxalaté frais de la malade.

80 (88)	70 (88)	94 (94)	34 (93)	85 (90)

2. Mélange de plasma malade et d'un plasma normal 50%.

valeurs en pourcents de la normale, entre () taux de F V calculé d'après la teneur en F V de chacun des plasmas.

–	88 (91)	100 (96)	47 (67)

3. Incubation à 37 °C pendant 1 heure.

du plasma malade et du plasma normal entiers,
du plasma malade et du plasma normal 1:10 en saline,
du mélange plasma malade-plasma normal entiers 50%

taux résiduel de F V après 60 minutes d'incubation; ce taux est exprimé en pourcents du taux avant incubation (= 100) quelqu en soit sa valeur absolue. Entre () valeur du plasma normal.

plasma incubé entier	–	13 (17)	59 (.0)	0 (30)	45 (43)
plasma incubé 1 10 en saline	–	16 (68)	87 (89)	1 (34)	
mélange 50% plasmas malade – normal entiers	37 (62)	16 (15)	.0 (34)	11 (22)	

Tableau IX

Effet anti-proaccélérine.

Matériel du 23 mars 1961 Fraction de plasma bovin précipitée à .0% sat. de sulfate d ammonium (riche en F V) TC en sec. Valeurs normales entre ().

Fraction bovine 1 partie + une partie de	non incubé	37 °C × 60 min
plasma .6 × 2 min	24,8 (24,3)	24,8 (24,9)
plasma Ba 56 × 2 min	27,3 (24,9)	27,8 (23,1)
plasma 56 × 30 min	28,0 (28,7)	28,5 (28,3)

(tableau I figure 2) Elle s associe à une dépression du groupe facteur VII facteur X qui n est qu apparente. En effet dans le sérum, après congélation ce dernier atteint des valeurs normales. Il s agit donc d un *défaut d'activation*, sans doute inhérent à la proconvertine (tableau X)

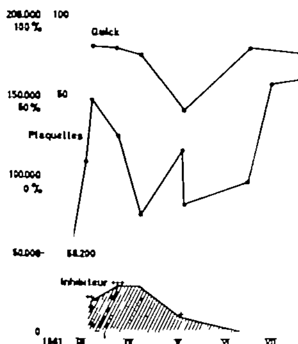


Fig 2 Evolution du taux des plaquettes, du temps de Quick et de l'inhibiteur

Tableau V
Défaut d activation du complexe F VII F X

prélèvement	plasma malade oxalaté frais en % de plasma N	sérum malade pris sur thromboplastine —30 C	jours en % de serum N
7 avril 1961	64	3	93
21 avril 1961	[illegible]	4	92
17 mai 1961	34	1	113

Discussion

Au cours de cette observation d ostéomyélosclérose polyglobulique se manifeste un syndrome hémorragique constitué par des ecchymoses, des hématomes, du purpura des membres inférieurs, le saignement prolongé des plaies, de minimes épistaxis. Il est apparu tardivement, après sept ans d évolution de la myélosclérose. Il est peu sévère, mais subit des poussées qui se rapprochent avec la lente aggravation de la maladie et qui ne répondent pas à des modifications précises de l'hémostase

Il dépend avant tout d une fragilité vasculaire sur laquelle vient se greffer peu à peu une thrombopénie. D abord modérée et inconstante celle-ci finit par être marquée et permanente mais toujours elle demeure instable Il y a parallélisme entre cette évolution et celle du tableau clinique et hématologique

A un certain moment au cours de ces oscillations thrombopéniques, apparait dans le sang un inhibiteur dirigé contre les plaquettes, associé à une labilité accrue de la proaccélérine (F V) à une hypoprothrombinémie vraie et à un défaut d activation de la proconvertine (F VII) Ces phénomènes rétrocèdent en quelques mois (fig 2)

Nous pensons que les oscillations thrombopéniques correspondent à des *crises de destruction plaquettaire*. Les plaquettes de notre malade ne manifestent pas de troubles fonctionnels elles sont normales. D un autre côté il est connu qu après splénectomie, au cours de la myélosclérose, s installe une thrombocythémie hémorragique et que le sang est envahi par des plaquettes aux formes très anormales, déficitaires dans le TGT lorsque l hyperplaquettose rétrocède ces monstres se font plus rares (7 obs. 1) Ces faits confrontés font croire que chez notre malade l accumulation dans la circulation des plaquettes malformées et des débris mégacaryocytaires est jugulée par des déplaquettisations périodiques, parfois brutales, ne laissant plus subsister que des éléments plus ou moins normaux.

L inhibiteur antiplaquettaire de notre malade se rattache à ces oscillations plaquettaires. Les inhibiteurs, apparus au cours de la fibrinolyse ne sont rien d autre que des produits de désintégration du fibrinogène et qui plus compétitifs, le remplacent dans la coagulation qu ainsi ils arrivent à empêcher (6) Dans notre cas, le matériel plaquettaire dégradé, libéré au cours d un paroxysme thrombocytolytique pourrait se substituer au FP 3 et de la sorte gêner la formation du procoagulant endogène. Une telle explication par compétition inhibitrice du facteur dénaturé nous paraît plus satisfaisante pour nombre d inhibiteurs pathologiques que la théorie qui voudrait en faire des anticorps. Notre hypothèse sur la genèse de l inhibiteur de notre malade est donc proche de celle que Loeliger (9) a proposé pour son cas elle n en diffère que sur ce dernier point de l origine immunologique

Notre observation touche à quelques problèmes que nous ne pouvons qu évoquer ici.

a) La présence de *cellules de Hargraves* dans cette ostéomyélosclérose typique dénote un processus d'immunisation. Des autoanticorps antiérythrocytaires, antileucocytaires et antiplaquettaires ont été décelés dans certaines myéloscléroses (11 obs. 3) mais non des anticorps antinucléaires. L'association d'un anticoagulant circulant à une hypoprothrombinémie et à une thrombopénie n'est pas non plus sans rappeler ce qui se passe dans un lupus érythémateux. Cependant, croyons-nous, l'inhibiteur et les cellules de Hargraves – d'ailleurs découvertes longtemps après sa disparition – ne sont pas directement liées et ceci est probablement aussi le cas dans le lupus érythémateux.

b) Le *syndrome hémorragique des myéloscléroses* est rapporté le plus souvent à la thrombopénie. Il est cependant des observations où coexistent les troubles plasmatiques de l'hémostase (12) et où un inhibiteur qui n'a pas été recherché, paraît quelquefois probable (8).

c) L'anticoagulant circulant de notre malade appartient aux *inhibiteurs antiplaquettaires* dont peu d'exemples ont été identifiés (13, 4, 5, 3, 5, 1, 2). Leur effet ne se limite pas au FP 3 (9) et la prothrombine peut apparaître comme un cofacteur de leur action ainsi que de celle d'autres inhibiteurs (10).

Résumé

Tard dans l'évolution d'une ostéomyélosclérose polyglobulique apparaît un syndrome hémorragique peu sévère. Il répond à une atteinte vasculaire et à une thrombopénie qui progresse par oscillations parallèlement à l'aggravation des autres symptômes. Succédant à une poussée de thrombopénie, un anticoagulant antiplaquettaire associé à une labilité accrue de la proaccélérine, à une hypoprothrombinémie vraie et à un défaut d'activation de la proconvertine évolue pendant plusieurs mois. La présence de cellules de Hargraves en petit nombre doit être signalée. Les auteurs présentent leur conception sur la pathogénie de cet inhibiteur et sur la signification de ces cellules de Hargraves.

Summary

At a late stage in the development of polycythaemic osteomyelosclerosis, a mild haemorrhagic syndrome occurred, with capillary fragility and thrombopenia progressing in spurts with the aggravation of the other symptoms. Following a thrombopenic episode, an antiplatelet anticoagulant factor appeared for several months, together with increased lability of proaccelerin, true hypoprothrombinaemia and deficient proconvertin activation. There were also a small number of LE cells. The significance of the latter is discussed, and also the authors' view of the origin of the inhibiting factor.

Zusammenfassung

In den späteren Stadien einer polyzythämischen Osteomyelosklerose entwickelt eine mittelschwere hämorrhagische Diathese mit zellulärer Fragilität und einer unter steten Schwankungen fortschreitenden Thrombopenie. Zugleich verschlimmert sich langsam das Gesamtbild des Leidens. Kurz nach einem Thrombosezustand erscheint ein wenige Monate andauerndes, gegen den Plättchenfaktor 3 gerichtetes Antikoagulans, zusammen mit erhöhter Labilität des Proaccelerins, echter Hypoprothrombinämie und einem Aktivierungsdefekt des Prokonvertins. LE-Zellen zeigen sich auch später in geringer Anzahl. Die Pathogenese des Hemmkörpers und die Bedeutung der LE-Zellen werden besprochen.

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Two New Thalassaemic Syndromes: a) Homozygous Alpha Thalassaemia. b) Cooley Like Disease due to Homozygous Elevated Hb A_2 Without Microcythaemia

By N Quattrin E. Dini and V Ventruto

The heterogeneity of thalassaemia is well known since Ingram and Stretton (1 2) distinguished two fundamental types, beta and alpha-thalassaemia, according to the genetic involvement of the beta- or alpha-globin chain. Beta thalassaemia is more frequent and is characterized by microcytosis, hypochromia, increased osmotic resistance, increased level of Hb A_2, whereas in alpha thalassaemia the level of Hb A_2 is normal or lower than normal.

Another condition, wich can be considered a variant of thalassaemia, described by Italian authors (3 4 5) is, really a trait and is characterized by normal blood picture and elevated level of Hb A_2. This condition which was designated as "elevated Hb A_2 without microcythaemia may be included in the group of those thalassaemic variants (6) to which the hereditary persistance of fetal haemoglobin Lepore Hb and Pylos Hb also belong

In a previous report (7) beta thalassaemia, alpha-thalassaemia and elevated Hb A_2 without microcythaemia were considered to be present in Campania in the following rate respectively 72 %, 20 % 8 %. Interest is growing in the study (8 9 10 11 12, 13 14 15) of the three conditions because of their possible combinations and genetic patterns (table III) The present paper describes two new syndromes due to homozygosity of thalassaemic genes.

Case I (table I and fig. I) De M. Addolorata, 6 years old, born and living in Naples. Her father, mother two brothers, maternal aunt and uncle are affected by alpha-thalassaemia. The child during her first year of life, had long period of vomiting due to functional pyloric stenosis. She came the first time to our observation when she was two years old severe anaemia and slight enlargement of spleen and liver were present.

Myelobiopsies. Tibial punctures were performed when the pat. was 3 and 5 years old. In both occasions the punctures showed marked hyperplasia of the erythroblastic

FAM DE MA

1 2 3 4 5 6 7

Hb$_H$ 2.4% Hb$_H$ 1% Hb$_H$ ... Hb$_H$ ... Hb$_H$ ... Hb$_H$ ... Hb$_H$ ...

alpha-thalassaemia

alpha-thalassaemia (homozygous) + Hb Bart

normal

dead

not tested

Fig 1 Pedigree of the Family De M. Seven patients are carriers of alpha-thalassaemia one (No. 6) is homozygous for alpha-thalassaemia.

cells with delayed maturation curve. HOTCHKISS *reaction* on the smears was negative as far as the erythroblastic series was concerned. *Electrophoretic pattern of Hb* showed great amount of Hb Bart (18.8) increased Hb F (9.2%) and small amount of Hb H (figs. 2 and 3) I that time this case was diagnosed as severe anaemia due to homozygous state of the alpha-thalassaemia gene (12).

Since her first admittance to the hospital the child and her parents were extensively investigated it could be ascertained that Hb Bart and Hb H were no more present and Hb F had returned to normal levels when pat. was 3½ years old.

The *X-ray examination of bones* did not show any abnormal findings when the baby was three however when she was six, an enlargement and deformation of the skull bone was observed.

Investigations of *glucose-6-phosphate-dehydrogenase* and *pyruvico-kinase* gave normal values.

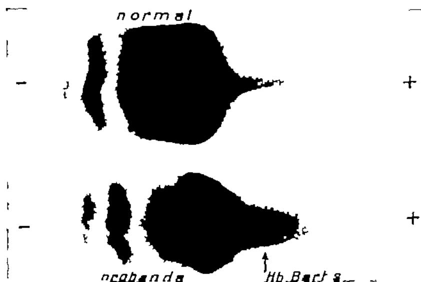


Fig. 2. Paper electrophoresis Hb pattern (pH 8.6) of case 1 at 2 years of age (above paper electrophoresis Hb pattern of normal subject)

The baby during four years was transfused repeatedly (10 liters of blood); the anaemia, however did not improve and the child shows now somatic hypoevolutism (fig. 4). Transient results were obtained by long treatment with Desferrioxamine B.

Discussion

In this case we deal with a condition of severe thalassaemic anaemia. This progressive disease, however differs from Cooley's anaemia because Hb Bart's and Hb H were present during the first years of life whereas no erythroblastemia, very increased Hb F bone changes, or marked hepatosplenomegaly were recorded. Moreover in this case, it is to be emphasized that the parents are carriers of the alpha-thalassaemia trait which was transmitted in heterozygotic form to the two sisters of the patient. In this case, we believe there is an interaction between the two alpha thalassaemia genes, therefore the disease is due to the homozygotic state of alpha thalassaemia. We would like to point out this finding insomuch as, contrarywise to previous opinions (13 14 15) it can be inferred that the homozygotic state of alpha thalassaemia may also represent a syndrome which is compatible with life this conclusion entitles us to support the idea that the homozygotic state of alpha thalassaemia has not, necessarily the letal outcome of a fetal erythroblastosis with ascites, described in the Chinese by Lie Injo Luang Eng (15) Although

Table I

Name	Age years	Sex	Hb	R.B.C. mil.	C.I.	M.C.V. [illegible]	Sanguel (0.4 [illegible])	Reticulocytes %	Red Cell Morphology A	P	M	H	Hb A_2 %	Hb F %	Index [illegible]
C. L. (1)	43	w	66	4.5	0.73	61	11	2.3	+	—	+++	++	2.5	—	—
C. V. (2)	38	m	86	7.3	0.58	55	32	0.8	++	+	+++	+++	2	—	150
C. C. (3)	27	w	74	6	0.60	00	17	0.7	+	—	—	++	2.3	0.6	170
Dr M. G. (4)	30	m	98	6.2	0.78	76	7.4	0.8	—	—	—	—	2.6	0.8	210
Dr M. F. (5)	1	w	90	6.5	0.69		42	0.4	+	+++	+	+++	2.8	1.21	130
Dr M. V. (case 1) (6)	2	w	40	3.9	0.51	45	28	6.3	++++	++++	+++	++++	2.7	9.2	161
Dr M. M. (7)	4	w	82	6.6	0.62	—	22	0.2	+	++	+	+++	1.5	1.22	133

A = Anisocytosis, P = Poikilocytosis, M = Microcytosis, H = Hypochromia. Hb Bart [illegible] 18.8

Table II

	M.A., Case 2			F[illegible]			M[illegible]			1. Brother		2. Brother
Date	22/3	26/5	10/9	2/4	14/5	29/10	26/3	14/5	23/10	14/5	29/10	14/5
Hb	20	60	20	84	88	96	86	68	74	105	102	102
R. B. C. mil.	1.2	3.2	1.6	4.6	4.9	5.4	5.1	4.5	5.1	5.7	6.3	5.5
C. I.	0.87	0.94	0.62	0.91	0.98	0.88	0.84	0.75	0.72	0.93	0.76	0.92
W. B. C. × 10^3	7.8	5	5.1	—	—	9.4	—	—	5.8	—	7.6	—
Reticulocytes, %	16	1	—	1.2	1.4	0.7	0.5	0.9	0.6	1.2	0.5	0.8
Erythroblasts (ort.)	2.5	0.5	3	abs.	abs.	abs.	abs.	abs.	bs.	abs.	b	abs.
P. C. V. %	13	18	—	—	—	50	36	29	—	45	—	42
M. C. V. cu μ	93	72	—	—	—	93	71	65	—	80	—	77
Osmotic fragility max.	0.31	0.32	—	—	—	—	—	—	—	—	0.32	—
(Hamburger Viola) min.	0.48	0.46	—	—	—	—	—	—	—	—	0.48	—
Sanguel (0.4 [illegible])	0.8	1.3	—	11	9.8	5.5	1.2	2.6	0.5	4.8	0.3	1.8
Proteinaemia, g	5.5	—	—	—	7.5	—	—	8.1	—	—	—	—
Hb A_2	5	8	4.8	7	—	5.5	7.8	7.4	6	2.9	3.8	1.8
Hb F	n. q.	n. q.	n. q.	n. q.	—	n. q.	n. q.	n. q.	n. q.	n. q.	n. q.	n. q.
Anisocytosis	+++	++	+++	+	+	+	+	+	abs.	abs.	abs.	+
Poikilocytosis	+++	++	+++	abs.	+	+	abs.	bs.	abs.	+	bs.	
Polychromasia	++	+	+	abs.	abs.	abs.	bs.	bs.	bs.	abs.	abs.	abs.
Target cells	abs.	abs.	abs.	abs.	++	abs.	abs.	abs.	bs.		abs.	+
Microspherocytes	+		+	abs.	abs.	abs.	abs.	bs.	bs.	abs.	abs.	bs.

[illegible]



Fig. 3. Paper electrophoresis Hb pattern (pH 7) of case 1 at 2 years of age. Comparison with paper electrophoresis Hb pattern in normal subject (above).

the syndrome we described is a severe one its final consequences cannot be predicted yet

Case 2 (table II). M. Anna, 39 years old, married born and living in S. Lucia di Serino (Avellino). Her father mother and brother show incomplete signs of thalassaemia. Four other adult brothers were not investigated because they are abroad. Since her early childhood the pat. showed sallow (pale and yellowish) colour of the skin. At 12 years of age she started to suffer from dyspeptic troubles. Her menstruation started when she was 13 and continued regularly.

At 37 she married a man in apparent good health she had no pregnancies. The physical examination shows a woman, 139 cm tall with body constitution under the normal. Her "facies" is typical for Cooley's disease (fig. 5) and her general condition poor. The skin and mucosae are of pale icteric colour. The first tone is impure (parophonic) at the apex. Blood pressure 115/55. The pulse is weak with 115 beats per min. Liver and spleen are markedly enlarged, but no swelling of the superficial lymph-nodes is appreciable.

Blood findings (see also table II and fig. 6). Hanger, Takata-Ara, McLagan and Wunderly cocolloidity tests were positive (+ +); bilirubin blood level 2,2 mg per 100 ml; iron blood level 140 γ%. Sickling test negative. Wassermann test negative. Urine without particular findings. Blood group and type A Rh positive. Blood N.P.N. 0.30‰, blood sugar 0.94‰.



Fig. 4 Photograph of case 1 (right) and of another girl of the same age (6 years).

X-ray examination of the bones (fig. 7) Hair-on-end" aspect of the skull, agenesia of the frontal and sphenoid sinuses.

Sternal puncture smears are rich in cells and clusters. The principal finding is the severe involvement of the erythroblastic system, because of the marked hyperplasia and profound troubles of maturation (fig. 8) Many cells show 3 and 4 nuclei or morula-like nucleus Pro-erythroblastic cells and macro-erythroblasts show often characteristics of the megaloblasts. Mitoses are not frequent. Granulocytic cells are decreased in number their maturation curv is of the delayed type. A few lymphocytes are present. Megakaryocytes are slightly decreased in number and ery few of them build p platelets. Stromal tissue is rich with endothelial cells, fibroblasts and reticular cells engulfing blood pigment.

Plasma cells are ery scanty Erythroblastic cells reacted negatively ith *Heilmeyer's test*

Discussion

Evaluation of data in M Anna shows that some findings are typical of COOLEY s disease whereas others are not. As far as the



Fig. 5. Case 2. Typical COOLEY "facies



Fig. 6. Comparative paper electrophoretic Hb patterns of subject with COOLEY disease (above), of case 2 (middle) and of normal subject (below)

patient is concerned the following signs speak for COOLEY's disease characteristic facies marked hepatosplenomegaly severe haemolytic anaemia stunted growth, bone changes, ellipto- and schizocytosis of the erythrocytes with slight erythroblastaemia. Moreover it is to be pointed out the marked increase of Hb A_2 in the parents of the patient Certain characteristics, however which are always present in COOLEY's disease are lacking in the pat. i. e the pronounced microcytosis, the hypochromia, the increased osmotic resistance, the presence of target cells and finally the increase of Hb F which is a constant sign. On the other hand the picture of the bone marrow was not characteristic of the homozygous thalassaemia because of the macro-erythroblastosis with negative HOTCHKISS test. This test is usually positive in the erythroblasts of COOLEY's disease (16, 17 18) even in benign forms of the disease (19) Furthermore the age of the patient (39 years) is against the diagnosis of COOLEY's disease. As far as the patient's relatives are concerned the parents showed a marked increase of the Hb A_2 fraction but no hypochromia or other morphological and osmotic abnormalities on account of the erythrocytes. A brother had hyperglobulia and a level of Hb A_2 a little above the normal.

In order to classify the case there are 3 possibilities to be considered 1) Both the parents are thalassaemic with however incomplete genic expression 2) The parents are separately carriers of thalassemia and of elevated Hb A_2 without microcythaemia respectively 3) Both the parents are carriers of the trait of elevated Hb A_2 without microcythaemia.

1 If it is to be admitted that both parents are thalassaemic even though with incomplete genic expression it is not clear why the patient lacked some of the typical signs of COOLEY's disease and why chiefly no Hb F was demonstrable. It has been also recently reported (20) that typical COOLEY's disease (so-called intermediate type) may occur when the parents show an increase of Hb A_2 with larvated cytologic signs of thalassaemia. In these cases, however there is a rather big amount of Hb F

2 If on the other hand the patient is the genetic product of a double heterozygosity between the thalassaemic gene and the increased Hb A_2 gene the clinical characteristics of COOLEY's disease and the absence of Hb F cannot be explained. In COOLEY like syndromes, due to the association of increased Hb A_2 and thalassaemia a marked increase of Hb F is a constant finding



Fig. 7 Case 2. Radiogram of the skull. Deformation and thickening of bones.

whereas the typical "facies" and bone changes of COOLEY's disease are missing (10, 11)

3 Excluding the two preceding hypotheses, it is to be ascertained whether or not the patient is the product of homozygous high Hb A_2 without microcythaemia. Such a syndrome has not yet been described but is theoretically predictable by the crossing of two carriers of the increased Hb A_2 trait It is certain that the parents of our patient which were repeatedly investigated, and probably also a brother are carriers of elevated Hb A_2 without microcythaemia.

The diagnosis, for the described case of COOLEY's disease of the adult must be rejected since no thalassaemia major was ever described without increase of Hb F On the other hand all the double heterozygous conditions between thalassaemia minor and other

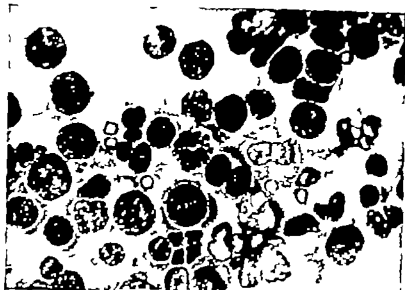


Fig. 8. Case 2. Bone marrow biopsy (May-Grünwald-Giemsa stain): macro-erythroblasts and morula-like nuclei.

haemoglobinopathies show an increase of the Hb F which, although variable, is always clear and as a rule, pronounced. We believe therefore to be entitled to conclude that this case of COOLEY like syndrome represents the first description of the disease due to homozygous Hb A_2 trait. In the picture of this COOLEY like syndrome there are to be amphasized the adult age of the patient the absence of microcytosis, hypochromia and increased osmotic resistance the medullary macroerythroblastosis with raspeberry like formations and negative HOTCHKISS's test and, finally the intriguing absence of Hb F

Conclusion

The two new thalassaemic syndromes described enriches our knowledge of the so-called COOLEY like anaemias. There is no doupt that these anaemias are apparently similar but really with different pathogenesis. To-day nearly 20 years after the description of the first form of COOLEY like anaemia i. e the sickle-cell-thalassaemic disease by SILVESTRONI AND BIANCO (6) we must conclude that new possibilities of genetic interactions may occur for these syndromes. In the field of the 3 thalassaemic genes already well known, five of the six possible interactions due to these genes are already known. The corresponding syndromes, including the one not yet known, are reported in table III

Table III
Interaction of the thalassaemic traits.

Genotype	Disease	Author	Year
Homozygosity for beta-thalassaemia	Thalassaemia major	COOLEY	1925
Double heterozygosity for beta-thalassaemia and elevated Hb A_2 without microcythaemia	Intermediate types. Severe form of the "macrocytic constitutional anaemia"	SILVESTRONI et al. (5)	1957
Homozygosity for alpha-thalassaemia	a) Erythroblastosis and hydrops foetalis	LIE INJO LUAN EWO (13)	1961
	b) Severe chronic anaemic syndrome of child	QUATTRIN et al. (12)	1961
Double heterozygosity for alpha- and beta-thalassaemia	COOLEY' anaemia (7)	MARINONE et al. (8)	1959
		BERNINI et al. (9)	1962
Homozygosity for elevated Hb A_2 without microcythaemia	COOLEY-like anaemia	QUATTRIN et al. (18)	1962
Double heterozygosity for alpha-thalassaemia and elevated Hb A_2 without microcythaemia	not yet described	—	—

Summary

Two new syndromes are described due to interaction of thalassaemic genes disease due to homozygous alpha-thalassaemia and disease due to homozygous elevated Hb A_2 without microcythaemia. The former disease differs from that described by Lie-Injo-Luan Eng because it occurs in early childhood and is not letal notwithstanding chronic severe anaemia. The latter is similar to COOLEY' disease differing from that, however in the very delayed course, the absence of microcytosis and increased osmotic resistance, the normal level of Hb F the absence in the bone marrow of microerythroblastosis with negativity of HOTCHKISS' test.

Résumé

Deux nouveaux syndromes dus à l interaction de deux genes de la thalassémie sont décrits une thalassémie alpha homozygote et une augmentation homozygote de la Hb A_2 sans microcythémie. La première diffère de la forme décrite par Lie-Injo Luan-Eng en ce qu elle apparaît dans la première enfance et qu elle n est pas letale malgré une anémie chronique sévère La deuxième ressemble la maladie de COOLEY mais diffère cependant de celle-ci par son décours tres prolongé, l'absence de microcytose et de résistance osmotique augmentée, par son taux normal en HbF et par l'absence de microérythroblastose dans la moelle osseuse, accompagnée d'une réaction de HOTCHKISS négative.

Zusammenfassung

Es werden zwei neue durch Thalassämie-Gene bedingte Syndrome beschrieben eine homozygote α-Thalassämie und eine homozygote Vermehrung von Hb A_2 ohne Mikrozythämie. Erstere unterscheidet sich von der von Lie Injo Luan-Eng beschriebenen Form, indem sie in der frühen Kindheit auftritt und trotz einer schweren chronischen Anämie nicht zum Tode führt. Letztere gleicht der COOLEY-Anämie, von der sie jedoch abweicht durch einen stark protrahierten Verlauf, das Fehlen von Mikrozytose und erhöhter osmotischer Resistenz, den normalen Gehalt an Hb F und das Fehlen der Mikroerythroblastose im Knochenmark bei negativer HOTCHKISS-Reaktion.

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Three Inherited Intra Erythrocytic Defects: Hereditary Spherocytosis, Hb S and Hb C*

By Robert B Thompson and Mason G Robertson

With the vast number of human hemoglobins in existence, it is not unusual to read reports of 2 or 3 intra-erythrocytic defects. The combination of hereditary spherocytosis with other hereditary blood dyscrasias is theoretically possible, but up to now only a few instances of hereditary spherocytosis associated with other traits have been identified. These are hereditary spherocytosis with sickle cell trait (6 2, 5) and with hemoglobin S and thalassemia (1) Individuals heterozygous for mutant genes at the locus determining the beta-polypeptide of hemoglobin particularly those with hemoglobin S and C are not common however the simultaneous occurrence of this anomaly with hereditary spherocytosis is distinctly rare.

Recently we had the opportunity to study a patient exhibiting the combination of hemoglobin S hemoglobin C and spherocytosis. The patient's mother had died at a very young age after having had 8 children. Because of evidence to be presented we strongly suspected that she was simultaneously carrying the gene for spherocytosis and hemoglobin S The maternal uncle had a splenectomy for hereditary spherocytosis. Of the 8 siblings only 5 survived and 2 of these were available for study One sibling an older brother was reported to have hemoglobin S-C disease with a history of splenectomy for spherocytosis.

The purpose of this paper is to report a family with the combination of hereditary spherocytosis and hemoglobin S + C and discuss the hematologic picture in this patient.

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* Supported by Institutional Grant UMC #5006—1—801—FR—05066—01 and UMC #5510—NIH AM 07943.

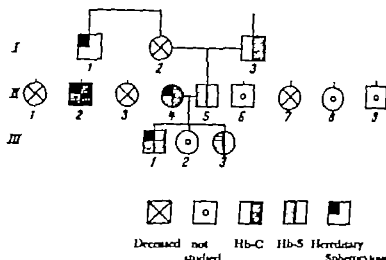


Fig 1 Pedigree of Family L. S. possessing hereditary spherocytosis, Hb S and Hb C.

Methods

Routine methods were used for the determination of hemoglobin, hematocrit, red, white and reticulocyte counts (7). Smears of peripheral blood stained with Wright stain were studied to determine abnormalities of red cell morphology A cell as considered to be spherocyte if it was small in diameter as compared to the other cells and was homogeneous and intensely stained.

The osmotic resistance of the red cells was determined by using dilutions of sodium chloride ranging from 0.24 to 0.80 gm %. The amount of hemoglobin present in the centrifuged hemolysate was measured at 540 mμ using Coleman Junior Spectrophotometer

Hemoglobin solutions were prepared by washing erythrocytes triply ith isotonic saline and hemolyzing with an equal olume of distilled water and 0.5 olume of toluene. The mixture was shaken for 20 minutes, frozen for two hours, thawed and recentrifuged at 2000 rpm for 10 minutes. The supernatant was aspirated and the remaining hemoglobin solutions filtered through Whatman No. 1 filter paper The lear hemolysate was subjected to electrophoresis on starch gel prepared as outlined arlier (3). The solubility of the hemoglobin samples in reduced state was studied by the method of Itano (4) and all hemoglobins subsequently designated as Hb S showed decreased solubility in the reduced state

Case Report

A 23 ear old colored female was referred to a hematologist by her local physician in October 1963 because of anemia of many years duration. She had three pregnancies leading to liv births During the pregnancies she was given iron and received several units of blood because of anemia during the postpartum period.

The patien ga history of ha ing had jaundice at the age of 10 ith recurring bouts of arthritis in her elbows, shoulders and knees coming on about twice a year She stated tha brother had been diagnosed as ha ing hemoglobin S C disease and spherocytosis for which splenectomy was performed. The patient mother died at ery young age after ha ing had 8 children. Only 5 of the patient siblings survived and none of the others part from the brother reported abov had any evidence f anemia A maternal uncle had splenectomy for spherocytosis.

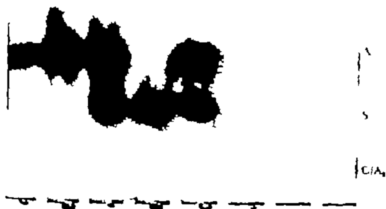


Fig. 2 Electrophoresis for family of L. S., on starch gel t pH 8. 1 Benzidine stain.

Table I
Hematologic data on families, 2 cases of hereditary spherocytosis and 3 cases of Hb S + C disease.

Case	Sex	Age	Hb phenotype	Hb gm %	Hct. %	RBC 10^6/cumm	MCV μ^3	MCH $\mu\mu g$	MCHC
I-3	M	54	AC	15.2	46	4 13	111	37	33
II-2	M	25	SC	9.5	30	3.40	88	27	31
II-4	F	23	SC	9.6	31	3.47	89	27	31
II-5	M	23	AS	13.2	42	4.92	85	27	31
III-1	M	9	SS	9.3	35	4.25	82	22	26
III-3	F	7	AS	12.0	40	4 85	82	24	29
Hb S + C (3 cases)				9.4	30	3 20	95	29	31
Hereditary spherocytosis (2 cases)				9.3	27	3.37	80	27	34
Normals (12 cases)				13.5	43	4 80	90	30	31

Physical examination revealed well developed, thin, small adult Negro woman in no acute distress. Temperature was 99 °F blood pressure 130/85, pulse 80 and there was no icterus. Abdominal examination revealed markedly enlarged spleen about 3 cm below the left costal margin with clear-cut splenic notch.

Hematologic investigation revealed hemoglobin of 10.6 gm %, hematocrit 30%, white blood count 6,750 with differential of 60% neutrophils, 34 lymphocytes, 3% monocytes, and 3% eosinophils. The platelet count was 208,000, reticulocyte count 1.9%. Sickle cell preparation by metabisulfite method was negative, direct Coombs negative, SGOT 14 units, SGPT 6 units and bilirubin 1 mg% total

The pertinent hematologic data pertaining to the patient father (I 3), the patient' brother (II-2) the patient under study (II-4), her husband (II 5) and two of the patient' children (III 1 and III 3) are presented in table I Additional data pertaining to the morphology of the erythrocytes and the osmotic fragility of the erythrocytes is presented in table II

Table II

Peripheral smear

Case	Hb phenotype	Hb solubility	hyperchromia	microcytosis	target cells	Osmotic fraction 10%	30%	50%
I 3	AC	93	1+	2+	slight	0.52	0.43	0.38
II 2	SC	40	2+	3+	2+	0.59	0.52	0.50
II-4	SC	43	2+	3+	2+	[illegible]	[illegible]	0.49
II 5	AS	[illegible]	1+	1+	neg.	[illegible]	0.47	0.41
III 1	SS	20	2+	2+	slight	0.60	[illegible]	0.40
III 5	AS	61	1+	1+	neg.	0.51	0.48	0.43
Normals (average 10 cases)						0.50	0.46	0.4[illegible]
Hereditary spherocytosis (2 cases)			slight	4+	slight	0.71	0.61	0.57
Hb S–C (3 cases)			2+	2+	+	0.53	0.44	0.38

Referring to the family pedigree (fig. 1) hematologic data (table I and II) and electrophoresis (fig. 2) we would like to make the following comments.

Case I 3, the father of the patient under study a 54 year old colored male, was entirely asymptomatic and apparently healthy. This hematologic data showed that he was heterozygous for hemoglobin C.

Case II 2, the brother of the patient under study was a 25 year old colored male who had previously had a splenectomy for hereditary spherocytosis. Electrophoretic examination of his hemoglobin disclosed that he was heterozygous for hemoglobin S and C.

Case II-4, the patient under study is a 23 year old female whose case history is presented above. Hematologic data revealed a combination of hereditary spherocytosis with hemoglobin S and C.

Case II 5, the husband of the patient under study an asymptomatic 23 year old colored male whose hematologic data show that he was heterozygous for hemoglobin S.

Case III 1 a 9 year old colored male, the son of the patient under study presented with a history of recurrent bouts of jaundice, joint pain and anemia. Hematologic data showed the combination of hereditary spherocytosis with homozygous hemoglobin S disease.

Case III 3, the youngest daughter of the patient under study a 7 year old colored female was asymptomatic. Hematologic data showed heterozygosity for hemoglobin S.

Discussion

If we analyze the hematologic data and the results of the genetic study we can suppose that the mother (I—2) of the case under study had a combination of hereditary spherocytosis and hemoglobin S. Then the marriage with a carrier for hemoglobin C resulted in two offsprings with the combination of the gene for hereditary spherocytosis and the double heterozygosity for hemoglobin S and C.

The findings showing the presence of the gene responsible for hereditary spherocytosis in Cases II 2, II-4 and III 1 are as follows: Marked spherocytosis and microcytosis in the blood smear (fig. 3) increased osmotic fragility a low normal MCV



Fig. 3 Peripheral blood smear on case II-4 (L. S.) Notice target cells and spherocytes. Wright stain, × 990.

The findings showing the presence of the genes for hemoglobin S and C are based upon (a) electrophoretic pattern, (b) reduced hemoglobin solubility (c) the presence of 2+ hypochromia and target cells in peripheral smear and (d) the presence of hemoglobin C trait in the father

The oldest sibling III 1 of the propitus disclosed the genes for spherocytosis and homozygous hemoglobin S disease. The findings of spherocytes in the peripheral smear with a biphasic osmotic fragility test showing increased fragility at high concentrations of saline and decreased fragility at low concentration plus the electrophoretic pattern and the presence of hemoglobin S genes in both mother and father would tend to confirm this diagnosis.

One in 1 000 Negroes has sickle cell hemoglobin C disease and although there are no good figures available the frequency of the hereditary spherocytosis is probably in the order of 1 in 10 000. Therefore, this case does indeed represent a reasonably rare combination of events. It is of interest that the combination of spherocytosis with sickle cell hemoglobin C disease results in an entity no worse than either alone Moreover the presence of three entities would indicate that spherocytosis is non allelic with the hemoglobin beta structure locus. This has already been noted by Cohen et al. (1)

Acknowledgements. The authors wish to express thanks to Mr. PAUL RAY for photographic assistance, to Mrs. LAMAR and Mrs. ODOM for technical assistance and to Mrs. L. MADDOX for secretarial aid.

Summary

A patient presenting a combination of hereditary spherocytosis with Hb S and Hb C is described. The genetic study showed that the father was heterozygous for Hb C and indirect evidence for the deceased mother suggested that she possessed the genes for hereditary spherocytosis and Hb S. The patient and one brother showed the same hematologic picture, which was not as severe as either disease state alone. Comments are made on the incidence of the hereditary spherocytosis alone and in combination with Hb S and Hb C and evidence for non allelism is presented.

Résumé

Rapport du cas d'un malade présentant une combinaison de sphérocytose héréditaire Hb S et Hb C. L'étude génétique relève que le père était un hétérozygote pur pour la Hb C cependant que la mère défunte possédait les gènes de la sphérocytose héréditaire et de la Hb S. Le malade et un frère présentent le même tableau hématologique, tableau qui est cependant moins grave que celui des deux maladies prises séparément. L'incidence de la sphérocytose héréditaire seule et en combinaison avec la Hb S et la Hb C fait l'objet d'un commentaire. Le non-allélisme de l'hérédité semble évident.

Zusammenfassung

Es wird über einen Fall berichtet, der eine Kombination von hereditärer Sphärocytose mit Hb S und Hb C aufwies. Die genetische Untersuchung ergab, dass der Vater heterozygot für Hb C war, während die verstorbene Mutter vermutlich die Gene der hereditären Sphärocytose und des Hb S besass. Der Patient und ein Bruder [illegible] dasselbe hämatologische Bild auf, das weniger schwer war als jede der beiden Krankheiten für sich. Die Häufigkeit der hereditären Sphärocytose allein und in Kombination mit Hb S und Hb C wird besprochen, und es wird auf den nicht allelen Erbgang hingewiesen.

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Acta haemat. 32: 239–249 (1964)

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Haemoglobin Bart's and H in a Swedish Boy

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In 1958 AGER AND LEHMAN (1) described a boy four weeks old, who had a thalassaemia like disease. Electrophoresis of the haemoglobin disclosed a fast moving component, which was called haemoglobin "Bart's". A similar and probably identical haemoglobin had earlier been described by FESSAS AND PAPASPYROU (7). Up to now all children with clinically detectable disease and with haemoglobin Bart's seem to have descended from Mediterranean or non-Caucasian nationalities. The following is a report of an infant boy in whom a transition from haemoglobin Bart's to haemoglobin H could be traced. The parents were healthy and of pure Swedish ancestry. The report includes special studies on the identification and nature of the abnormal haemoglobins.

Case History

L. P., boy born on July 31st, 1961. He was the first child of healthy parents, and was born two weeks before term after an uneventful pregnancy. At birth the amniotic fluid was meconium-stained and the infant slightly cyanotic; regular respiration started after ten minutes. Despite suction of the airways, the boy remained rather blue, and was given oxygen. The placenta, 565 g, was macroscopically normal. At 12 hours of age a paediatrician examined the boy, who was grunting, moderately cyanotic, slightly oedematous, and had generalized petechial haemorrhages. There was no icterus. The external ears were low-set and deformed, abundant skin folds were present at the back of the neck, the testes were undescended and the scrotum hypoplastic. The liver margin was felt 1½ cm and the spleen 3 cm below the costal arch. On X-ray examination the lungs were found to be normal, but the heart appeared enlarged, and there was ascites. Laboratory tests revealed a haemoglobin concentration of 20.3 g per 100 ml, thrombocytopenia, reticulocytosis, and a great number of nucleated red cells in the peripheral blood. The haematological findings are reported separately below and in table I. There was no blood-group incompatibility between mother and child, and Coombs direct test was negative. Septicaemia, meningitis, syphilis, toxoplasmosis, listeriosis, and cytomegalic inclusion-body disease could be excluded by appropriate investigations.

During the following days, the boy's condition improved spontaneously. The oedema and ascites disappeared. The size of the liver, spleen, and heart returned to normal. The boy was inactive, however, and had to be fed by tube. The reticulocyte and

thrombocyte counts became normal (table I). At the age of one week coccoid inclusion bodies were found in the erythrocytes.

The boy remained in the hospital until the age of 6 months, and returned for follow-up examinations several times during the following years. At the age of 22 months he was still extremely hypotonic, and all original stigmata were still present. His mental and motor development at this time corresponded to that of an infant about 6 months old.

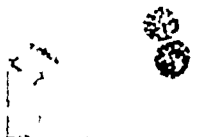


Fig. 1. Erythrocyte inclusion bodies after incubation with brilliant cresyl blue.

Laboratory Investigations

The pertinent haematological findings are summarized in table I. The *haemoglobin concentration* was high in the neonatal period, and decreased to 9.0 g/100 ml during the first four months, after which time slow increase was noted. The erythrocytes showed several interesting abnormalities. From the neonatal period onwards coccoid *inclusion bodies* could alw y be found after incubation with brilliant cresyl blue (fig. 1). At birth about 60,000 *nucleated red cells* were found per cu.mm. of peripheral blood. During the first two weeks of life the number slowly decreased. From about one month of age numerous *target cells* were noted, amounting to about 10% of all erythrocytes. During the neonatal period the haemoglobin content and haemoglobin concentration of the red cells did not appear abnormal, but at about the age of 3 months the cells became hypochromic and macrocytic. The number of *reticulocytes* was abnormally high during the first day of life (15) but decreased rapidly during the neonatal period. From the age of three months, occasional high counts were noted (8.4 and 9.2%) but during the second year of life the reticulocyte counts became normal. *Bone marrow smears* were ob tained in the neonatal period. Except for ery active erythropoiesis, the study no abnormalities.

The *mechanical resistance* of the red cells was normal. The *neutral haemolysis* was raised at 11 day of age, but became normal later on. T *concentration* in serum was studied at 1 4 6 and 9 months of age, the 25, 56 and 55 mg per 100 ml respectively Icterus was never noted *bilirubin* concentration was 1.0 mg/100 ml serum. The concentra *cholesterol* and *total lipids* were found to be normal. On paper el protein pattern seemed normal. The *chromosomes* of bone marrow pattern. The *electroencephalogram* was normal at the age of 4 mon

Haemoglobin Investigations

Methods

Supravital staining with brilliant cresyl blue: Equal

1 solution of brillian cresyl blue were incubated for

were then prepared, and the incidence of red cells with inclusion bodies and of reticulocytes per thousand red cells was calculated. As staining of the bodies in the different cells varied in intensity exact enumeration of these cells was difficult.

Preparation of haemoglobin. Heparinized blood was washed twice with normal saline within 1–2 hours of sampling. Two ml of the washed corpuscles were diluted with 2.4 ml of distilled water. After addition of 1 ml of toluene the sample was shaken for 3 minutes and centrifuged for 20 minutes at 2500 rpm. The clear bottom layer of haemoglobin was recovered and treated by bubbling with carbon monoxide during several minutes. If not examined immediately the samples were stored at −20° C.

Electrophoresis of the haemoglobin solutions was performed with granular starch as supporting medium (13) in barbital buffer (pH 8.6, ionic strength 0.05). In some experiments phosphate buffer of pH 7.5 and of varying ionic strengths was used (3). After electrophoresis the different fractions were cut out and eluted by suction-filtration and washing with normal saline, quantitated by spectrophotometry at 540 mμ, and expressed as per cent of the total haemoglobin.

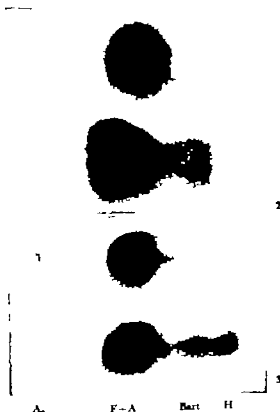


Fig. 2. Starch slab electrophoresis of haemoglobin from a normal adult (above) and from the patient at the age of 1 month (below).

Fig. 3. Starch slab electrophoresis of haemoglobin from a normal adult (above) and from the patient at the age of 3 ½ months (below).

Table I
Haematological findings in the patient.

Age	Haemoglobin g/100 ml	Reticulocytes %	MCHC %	MCH $\mu\mu g$	MCV μ^3	Platelets 1000 per mm³	Haemoglobin components, A₂	Bart	H	Red cells with inclusion bodies	[illegible]
1 day	20.3	15	29	34	116	43	—	—	—	—	
11 days	20.3	2.8	31	29	92	120	trace	19	0	5	
1 month	13.6	1.0	29	27	92	232	trace	16	0	8	
1 m 10 d	10.8	1.0	27	25	90	274	trace	+	0	18	
2 m 25 d	9.4	3.0	26	22	86	350	trace	12.5	5	49	
3 m 16 d	9.0	8.4	26	21	80	360	1.6	10	9.5	51	1
5 m 9 d	9.1	4.2	24	19	80	412	2.2	4.5	7.7	46	
9 m 8 d	10.2	9.2	25	24	95	430	[illegible]6	0	10.1	3[illegible]	
1 y 4 m	11.9	2.4	28	24	86	236	2.0	0	8.[illegible]	39	
1 y 10 m	10.8	2.0	28	23	79	98	2.3	0	3.9	49	

Haemoglobin Bart found, not quantitatively estimated. MCHC = mean cellular haemoglobin concentration. MCH = mean cellular haemoglobin content. MCV = mean cellular volume.

Alkali-denaturation test. As Hb A and Hb F show poor separation in electrophoresis as described above the latter component was quantitated by the alkali-denaturation test as described by SINGER et al. (24). The alkali resistance of isolated haemoglobin fractions was evaluated by the same method.

Ultraviolet spectroscopy. The ultraviolet spectral absorption curves of isolated haemoglobin fractions was measured with the aid of a Beckman DB spectrophotometer.

Results

The first characteristic to suggest the possibility of a haematological disorder was, as is usually the case, a morphological one, namely the presence of target cells and the appearence of red cells with inclusion bodies on supravital incubation with brilliant cresyl blue. The frequency of these inclusion cells (about 5 % in the neonatal period) showed a slow increase during the first six months of age.

The first electrophoretic investigation (11 days) showed a main fraction consisting of poorly-separated Hb A and Hb F and traces of Hb A_2, and an abnormal fast-moving component constituting about one-fifth of the total haemoglobin content. This abnormal component showed a slow but steady decrease during the first few months (table I and fig. 2). When the boy was aged 2.5 months a second fast-moving component appeared, with slightly higher mobility than the first (fig. 3). To start with this faster moving component comprised about 5 % of the total haemoglobin, but subsequently showed a slow increase, balancing a simultaneous slow decrease in the less-fast abnormal component. When the boy was 9 months of age this less-fast component was no longer demonstrable, while the faster one had increased to about 10 %. Subsequently this component also diminished slowly and at two years of age amounted to about 4 % of the total haemoglobin.

Initially the Hb A_2 component was demonstrable only in traces. It then increased slowly to reach 2.5 % of the total haemoglobin, a normal figure, when the boy was [illegible] years old.

Identity of abnormal components. Owing to the rarity of abnormal haemoglobins in Sweden an electrophoretic comparison with known haemoglobin components was impossible here. Dr. FESSAS of Athens kindly did this for us, and found that the faster moving component migrated as Hb H and the less fast moving component as Hb

Bart. Our investigations provided further confirmation of this. Ultra violet spectrophotometry disclosed in the less fast moving component marked tryptophan band similar to that of Hb F whereas the faster moving component was more reminiscent of Hb A in this respect. It was also found that the less fast moving component was about four times more alkali-resistant than the faster one. All this indicates that the less fast moving component had the characteristics of Hb Bart and the faster those of Hb H.

When brilliant cresyl blue was added to the haemolysate before electrophoresis, the faster moving component became denaturated (precipitated) and remained at the application slit of the electrophoresis block, which corroborates the assumption that this component was identical to Hb H. The same applies to the positive correlation between the number of red cells with inclusion bodies and the amount of the fast moving Hb component.

Bergren et al. (3) have reported that under certain conditions Hb H may separate into two different isomeric, fast migrating components, as a result of changes in ionic strength and pH of the electrophoresis buffer, the more slowly migrating subfraction increasing in amount with increase in pH or with fall in ionic strength. In our case no tendency to isomeric splitting of Hb H was found on changes of pH or ionic strength.

Family Studies

The parents. The boy's father and mother had always been healthy. They were examined haematologically and by haemoglobin electrophoresis without any abnormal findings (table II). Careful search for coccoid inclusion bodies after incubation with brilliant cresyl blue was fruitless in both parents.

Table II

Haematological findings in the parents

	Father I	Mother R P
Haemoglobin, g/100 ml	14 5	14 0
Mean cellular haemoglobin concentration,	35	34
Reticulocytes, %	2 2	4 0
Serum iron, mcg/100 ml	74	85
Total iron binding capacity mcg/100 ml	304	292
Haptoglobin, mg/100 ml	1?0	160
Bilirubin, mg/100 ml	0 5	0 2
Osmotic resistance of red cells	normal	normal
Abnormal haemoglobins	not found	not found
Hb A_2	2 3	2 3
Hb F (Singer) %	0 6	0 6
Coccoid inclusion bodies	not found	not found
Target cells	not found	not found

Paternity. The boy's blood group was A BMNSs P(+) kk Fy(+) Jk(b) Rh( ) type Rh_1Rh_2 and the serum group was Hp(2 2) Gc(2 1) Gm(+). The father's blood group was A_1Ns P(+) kk Fy(+) Jk(+b+) Rh+ type Rh_1Rh_2, and the serum group was Hp(2 1) Gc(2 1) Gm(+). The mother's blood group was B MSS P(+) kk Fy(a—) Jk(a+b+) Rh(+) type Rh_1rh, and the serum group was Hp(2 1) Gc(2 1) Gm(a—). Statistical treatment of the data revealed that the probability of true paternity was 94.2%.

The authors are greatly indebted to Professor B. Broman, State Institute for Blood Group Serology, who performed the analyses.

The pedigree The boy ancestry seemed purely Swedish. The pedigree could be traced for at least three generations. There was no history of blood disease or of mental deficiency among the relatives. All of them had lived in the eastern middle part of Sweden.

Discussion

The haematological and haemoglobin investigations strongly suggest that the abnormal haemoglobin components encountered in the blood of this boy are identical to those named in current literature as Hb Bart s and Hb H Haemoglobin Bart s has been found in normal newborn infants, in infants and children with clinical disease and in infants stillborn or dying at birth.

Studies on *cord blood from unselected neonates* have revealed a varying incidence of Hb Bart s in the absence of other clinical or chemical abnormality The concentrations of Hb Bart s have varied between barely detectable up to 24–25 % (26) Hb Bart s was found in 3–5% of a series of Chinese infants (14 19 26) in Thai infants in 5 % (25) in Nigerian neonates in 10 % (9) in North American Negro infants in 4–7 % (20 22) and in Caucasian neonates from East Asia (26) and North America (20 22) in about 1 %. In all, the three last named studies included 464 white neonates, of which 4 had Hb Bart s. In Europe, two cases were found among 500 Greek infants (6) and a study of 3556 Italian babies (23) revealed 9 cases. Follow up examinations have been performed on some of these cases (6 14 20 22 23 25, 26) Hb Bart s was found to disappear between the ages of 3 days and 4 months, and was not replaced by other abnormal haemoglobins. A study of some of the parents has been performed (6 14 20 23 25 26) but no abnormal haemoglobins and no definite signs of thalassaemia have been found.

Hb Bart s associated with haematological abnormalities. In surviving infants Hb Bart s has been found together with Hb D (20) Hb E (25) and Hb S (11) The coexistence of Hb H and Hb Bart s has been noted also in older children and adults (6 10, 21) In most of these cases, hypochromic and microcytic anaemia was found. Haemoglobin Bart s has been demonstrated in hereditary elliptocytosis (20) and in glucose-6-phosphate dehydrogenase deficiency (26) but its presence was probably only coincidental. The association of Hb Bart s with thalassaemia has been much discussed since the first reports (1 7) describing infants with Hb Bart s and a thalassaemia like disease. The Hb A_2 concentration was not determined in these infants. In the first report 7 the mother had

thalassaemia major and the father thalassaemia minor but their Hb A_2 and Hb F concentrations were not reported. In the second paper (1) the parents were reported to have low MCHC and increased erythrocyte osmotic resistance but no increase in Hb A_2.

Haemoglobin Bart's associated with perinatal death Nin[illegible] ses of perinatal death associated with a clinical picture similar to h[illegible] d[illegible] ps foetalis and with "large amounts of Hb Bart's have been r[illegible] d in Indonesian-Chinese babies by Lie Injo Luan Eng et al [illegible] 17 18) The clinical findings included ascites, oedema [illegible] ment of the liver and in some cases, also of the spleen Pla[illegible]ntas that were examined were large and friable. Laboratory investigation failed to reveal iso-immunization. In all cases in which the peripheral blood could be studied, anaemia, reticulocytosis, numerous nucleated red cells, and target cells were found. After incubation with brilliant cresyl blue, intracellular crystals [illegible]ere formed in all cases in which the examination was made. Haemoglobin electrophoresis revealed large amounts of Hb Bart's, small amounts of Hb A, and, in 4 cases, the definite presence of Hb H In one additional case Hb H was probably present. These findings together with the findings of intracellular crystals make it probable that Hb H was present in at least 6 cases. In no case was the presence of Hb H excluded. Fourteen of the 16 parents two infants were twins) were examined no abnormal haemoglobins were found, and there was no increase in alkali resistant haemoglobin or in Hb A_2. Although many of the mothers were moderately anaemic, no definite conclusions could be drawn regarding thalassaemia.

When compared to the earlier reports on infants with haemoglobin Bart's, *the present case* shows definite similarities to the infants described by Lie Injo Luan Eng et al. The coexistence of Hb Bart's and Hb H in newborn infants has been reported only by these authors, and the main clinical signs of disease (oedema, ascites, enlargement of liver and spleen, and erythroblastosis without iso-immunization) are features in common between our case and those of Lie Injo Luan Eng There are, however also definite dissimilarities. The Indonesian-Chinese infants were severely ill, many were stillborn, and none survived the neonatal period. Hb Bart's constituted the major part of their haemoglobin, their placentas were grossly abnormal, and no thrombocytopenia or obvious malformations were reported.

The question of whether or not the clinical signs in our case and in those of LIE INJO LUAN ENG et al. may be regarded as signs of haemoglobinopathy must remain open for the time being. It seems possible however that the severity of the disease is correlated to the relative amount of Hb Bart's. In the Indonesian Chinese babies Hb Bart's constituted the major part of the haemoglobin, whereas in our case the relative concentration of Hb Bart's was only 20 %. In most symptomfree neonates with Hb Bart's the concentration of this haemoglobin has not exceeded 15 %. Available reports on cases with Hb Bart's seem also to indicate that the association of this with another abnormal haemoglobin may worsen the disease. In LIE INJO LUAN ENG's cases Hb H was probably always present. In the case described by TUCHINDA (25) Hb Bart's and Hb E were associated with the findings of numerous nucleated red cells, petechial spots, and hepatosplenomegaly during the neonatal period. Reticulocytosis and target cells were observed in a newborn with Hb D and Hb Bart's (20). In our case Hb H as evidenced by red cell inclusion bodies, was present from the neonatal period and subsequently Hb H was demonstrated by electrophoresis. In asymptomatic neonates with Hb Bart's on the other hand, the presence of Hb H has only been suggested by FESSAS's finding of a few inclusion bodies (6).

In most haemoglobinopathies the abnormal haemoglobin is also found in one or both of the parents. This is not the case when Hb Bart's or Hb H are concerned. The parents of most asymptomatic neonates with Hb Bart's showed no signs of haemoglobinopathy. The studies of FESSAS (6) alone have revealed a few cases of Hb H among parents of neonates with Hb Bart's. In other studies, however, a thalassaemia like disease has been found in some of the parents (1). This "thalassaemia" was not of the common type including a raised concentration of Hb F and or Hb A_2 (β-thalassaemia) but more like that described in Hb H families (5, 8) and called α-thalassaemia by INGRAM AND STRETTON (12). In α-thalassaemia the synthesis of α-chains is probably diminished, and β-chains and γ-chains are formed in excess. This is thought to give the formation of the tetramers β_4 (= Hb H) and/or γ_4 (= Hb Bart's) depending on the relative rate of synthesis of β- and γ-chains. On theoretical grounds, a homozygous α thalassaemia is supposed to be incompatible with life (2, 12) and LIE INJO LUAN ENG et al. (17) suggested that their cases were

in fact examples of homozygous α thalassaemia. In our case it is impossible to say anything definite about the genetics. The negative results of the haematological study of the parents do not fit in very well with homozygous disease in the patient. It may be noted however that in LIE INJO LUAN ENG's cases, the family studies were essentially negative. Furthermore the minimum [illegible] the diagnosis of α-thalassaemia trait are not establi[illegible] finding of Hb Bart's in the neonatal period in otherw[illegible] infants may in fact represent the only sign of the tr[illegible] Finally Hb H disease has been found in the Swedish p[illegible]pu[illegible] (8). For the time being we may suppose our patient's diseas[illegible] hereditary rather than acquired in early fetal life. The simultan[illegible]us occurrence of malformation and haemoglobinopathy is uncommon and it seems likely that in the present case the combination is fortuit[illegible]us.

Summary

A new-born full-term boy was found to be suffering from re[illegible]piratory distress, oedema, enlargement of the liver and spleen, petechial haemorrhages, and minor congenital malformations. Haematological investigation showed thrombocytopenia, reticulocytosis, and [illegible] great number of circulating nucleated red cells. There was no [illegible] and no evidence of iso-immunization. Intraerythrocytic inclusion bodies suggested the presence of Hb H, but up to the age of 3 months Hb Bart [illegible] the only abnormal component found on electrophoresis. From 3 to 9 months of age both Hb H and Hb Bart's were demonstrable. After 9 months only Hb H [illegible] found. The clinical condition of the infant improved during the first week of life. His mental development was slow and at 20 months of age was severely retarded.

Résumé

Chez un nouveau-né de sexe mâle [illegible] à terme se trouvèrent des signes d'insuffisance respiratoire, des œdèmes, un agrandissement du foie et de la rate, des hémorrhagies pétéchiales et des malformations congénitales mineures. U[illegible] examen hématologique démontra une thrombocytopénie, une réticulose et un grand nombre d'érythroblastes. Il n'y avait pas d'anémie ni [illegible] aucun signe d'isoimmunisation. Des inclusions intraérythrocytaires suggérèrent la présence de Hb H. Mais jusqu'à l'âge de 3 mois on ne décela comme composante anormale que de la Hb Bart, à l'électrophorèse. Du troisième [illegible] mois on trouv[illegible] aussi bien de la Hb H que de la Hb Bart, après le neuvième mois plus que de la Hb H. L'état clinique du nouveau-né [illegible] améliora durant la première semaine de [illegible]. Son développement mental fut très lent et était très en retard à l'âge de 20 mois.

Zusammenfassung

Bei einem am Termin neugeborenen Knaben fanden sich eine respiratorische Insuffizienz, Oedeme, Leber- und Milzvergrößerung, petechiale Blutungen und geringfügige kongenitale Mißbildungen. Die hämatologische Untersuchung ergab eine Thrombocytopenie, eine Retikulocytose und eine große Zahl zirkulierender Erythroblasten.

Eine Anämie oder Zeichen einer Isoimmunisierung lagen nicht vor Erythrocytäre Innenkörper erweckten den Verdacht auf das Vorliegen von Hb H, jedoch ergab die Elektrophorese bis zum Alter von 3 Monaten als einzige abnorme Komponente Hb Bart s. Vom 3. bis zum 9. Monat waren *Hb H* und *Hb Bart* nachzuweisen. Nach dem 9. Monat fand sich nur Hb H. Der klinische Zustand des Kindes besserte sich im Laufe der ersten Lebenswoche. Seine geistige Entwicklung erfolgte langsam und zeigte im Alter von 20 Monaten eine schwere Verzögerung.

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Basophil Leukocytes and Tissue Mast Cells in Newt, Diemyctylus viridescens*

By Ronald R. Cowden, Avdhesh Narain and George C. Beveridge

Both basophil leucocytes and tissue mast cells of mammals have been extensively studied by the more recently developed techniques of cytochemistry and cellular physiology, but the homologous cells in lower vertebrates have received scant attention. An accumulation of evidence now indicates that these two types of cells share some physiological functions in mammals but their histogenesis, nuclear morphology, and cytoplasmic ultrastructure are sufficiently divergent to classify them as different types of cells (13, 15, 16). In non-mammalian vertebrates, the nuclei of both types of cells are spherical in most instances, but little attempt has been made to distinguish between them on morphological grounds or on the basis of their chemical organization. For this reason a comparative cytological and cytochemical investigation was undertaken on the basophil leucocytes and tissue mast cells of the newt *Diemyctylus viridescens*. This animal was chosen because urodele amphibia represent the lowest vertebrate group in which both types of cells are present in reasonable numbers, and because of its ease of maintenance in the laboratory. The relatively large size of cells in this animal offers an additional advantage in a study of this nature.

Materials and Methods

The newts used in this study were obtained from a commercial source and maintained in aquaria. Peritoneal fluid was obtained by opening the abdomen from the ventral aspect taking care to avoid excessive bleeding, and aspirating small amounts of peritoneal fluid with a capillary pipette. Smears were prepared in the conventional

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* This work was supported by a grant from the U. S. Public Health Service (RG-10003-02) and a research career award to Dr. Cowden from the National Institute of Child Health and Human Development (5-K3-HD-1 [illegible]).

manner and air-dried. Blood was taken directly from the heart and [illegible] smears were prepared and air-dried. The smears were fixed in absol[illegible] [illegible] ethanol-acetic acid (3 1) or 10% formalin in absolute ethanol. Sections [illegible] [illegible] [illegible] and mesentary were fixed in ethanol-acetic acid, alcoholic [illegible] [illegible] neutral formalin. These were embedded in the conventional manner [illegible] [illegible] were prepared.

DNA was demonstrated in ethanol-acetic acid fixed [illegible] reaction in which an azure A-SO_2 reagent prepared according [illegible] substituted for the usual leuco-basic fuchsin Schiff reagent [illegible] [illegible] the acrolein-Schiff reaction for proteins was carried out [illegible] [illegible] reagent (3). Protein tyrosine was stained in similarly fixed prepa[illegible] [illegible] Smart' (12) modification of the Millon reaction. [illegible] [illegible] was exclusively employed for the demonstration of mucopolysaccharides [illegible] [illegible] tions were stained for 5 minutes in 0.5, 0.05 and 0.005 [illegible] toluidin blue [illegible] to develop basophilic metachromasia typical of acid mucopolysaccharides [illegible] at pH 0.3 was used for the detection of sulfated acid mucopolysaccharides [illegible] the paraldehyde-fuchsin method which, according to Spicer (14) [illegible] [illegible] highly sulfated acid mucopolysaccharides. Neutral mucopolysaccharides [illegible] [illegible] with adjacent glycol groups were demonstrated by the periodic acid-Schiff (PAS) reaction. Methanol fixed smears were stained with Wright['s] blood stain, and neutral formalin fixed tissues were sectioned and stained with the [illegible] [illegible] method.

Cell and nuclear diameter measurements were performed on 50 randomly encountered cells of each type using [illegible] 54 × Leitz oil immersion obj[ective] and Leitz [illegible] 5 filar micrometer ocular. Nuclear, cell and cytoplasmic areas were computed in arbitrary units using the standard equation for the area of an ellipse.

Results

In the sectioned material, mast cells were most frequently encountered in the abdominal mesenteries. They were only infrequently seen in other tissues. Neither basophil leucocytes nor tissue mast cells were found in the subcapsular granulopoetic layer of the liver. They were, however, observed in the spleen lying outside blood vessels and sinuses. Although developmental stages could not be clearly distinguished in the sectioned material, the spleen rather than the subcapsular layer of the liver was considered the principal site of basophil leucocyte production.

The tinctorial behavior of the granules of both basophil leucocytes and tissue mast cells was virtually identical after staining with methods for the demonstration of both proteins and mucopolysaccharides. They were stained by both the Millon reaction for protein tyrosine and the acrolein-Schiff reaction for protein. The granules of both types of cells were considerably more weakly stained than the cytoplasm of polymorphonuclear and eosinophilic leucocytes by the PAS reaction, and were completely unstained by the paraldehyde fuchsin method. Both types of cells were less deeply

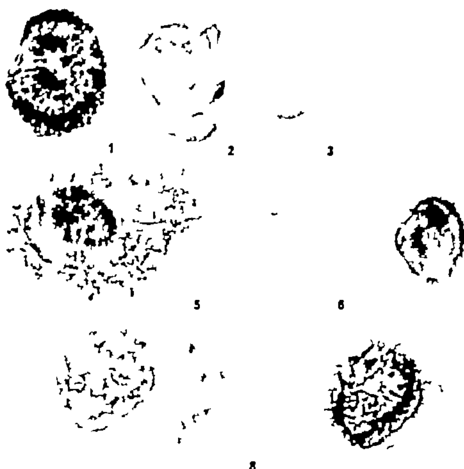


Fig 1 Basophil leucocytes and tissue mast cells of the newt (Diemyctylus viridescens) 600 1 Basophil leucocyte 0.5 toluidine blue 2 Basophil leucocyte pH 0.3 astrablau 3 Basophil leucocyte PAS reaction with green filter 4 Mast cell, pH 0 3 astrablau 5 Mast cell, 0.5 toluidine blue 6 Mast cell, PAS reaction with green filter 7 Basophil leucocyte Feulgen-Azure A acrolein-Schiff with red filter 8 Mast cell, Feulgen-Azure A acrolein-Schiff reaction with green filter 9 Mast cell, Feulgen-Azure A acrolein-Schiff reaction with red filter

stained by pH 0 3 astrablau than rat mast cells, but the newt mast cells were not stained as deeply as newt basophils. Both types of cells failed to stain with very dilute toluidin blue (0 005 / in 30 etha nol) The granules were weakly stained by toluidine blue at an order higher concentration and were deeply stained by 0.5 toluidin

blue in 30 / ethanol. In both instances the toluidin blue staining was largely orthochromatic.

As shown in fig 1 basophil leucocytes and mast c lls could be clearly distinguished on the basis of two morpholog al f rac teristics. Basophil granules were generally coarser and m r bl in size than mast cell granules, and mast cells possessed more cytoplasm than basophils. The results of nuclear n plasmic area measurements were computed as nuclear-cy ratios. These results are presented in the form of a hi togr fig. 2. The values were consistently higher in basophil l u indicating consistently less cytoplasm in these cells, and the ba phil nuclei were generally larger than mast cell nuclei The spread of values in this limited sample was much greater among the basophils than among mast cells.

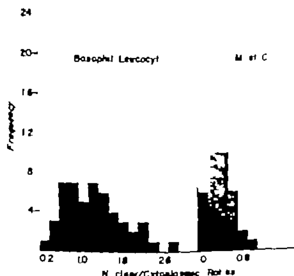


Fig 2

Discussion

The earlier accounts of non-mammalian basophil leucocytes and tissue mast cells (2 4 6, 7 10) have generally stressed the similarity of these cell types, and little information is currently available concerning their divergence in phylogeny KELLY et al. (9) were unable to find any consistent differences in tinctorial behavior after toluidin blue and astrablau staining between these

two types of cells in the horned lizard, but their published photographs indicated that there are morphological differences similar to those found in the newt between basophil leucocytes and tissue mast cells. The observed differences in granule size and nuclear/cytoplasmic ratio in these two types of cells indicates the existence of fundamental biological differences. A divergence between these two types of cells may now be considered to extend as far back in vertebrate phylogeny as the urodele amphibia.

Although marked differences between the mucoproteins of newt basophil leucocyte and tissue mast cell were not uncovered with the cytochemical methods employed, the cytoplasmic mucoproteins of these cells differ in some respects from those of homologous cells in mammals. As SPICER (14) has indicated there is a reciprocal relationship between the degree of staining obtained with methods for detecting sulfated acid mucopolysaccharides and the intensity of the PAS reaction. Staining in newt cells with methods for acid mucopolysaccharides are less intense than in homologous mammalian cells (1, 11) and this was not matched by a corresponding increase in PAS staining. This suggests that the concentration of mucopolysaccharide is lower in relation to than of protein in newt basophil and mast cell granules than in those of homologous mammalian cells. The results also suggest that the mucopolysaccharides of the newt cells are less sulfated than in corresponding cells in mammals. An extreme solubility of basophilic substances in lower vertebrate mast cells was noted by MICHELS (10) and this factor may have reduced the concentration of mucopolysaccharides in the preparations of newt cells.

Summary

Cytological and cytochemical investigations of basophil leucocytes and tissue mast cells in the newt, *Diemyctylus viridescens*, have indicated that these two types are morphologically distinct. Basophils contain larger granules and less cytoplasm than mast cells. Cytochemical methods for proteins and mucopolysaccharides failed to disclose any significant difference between the two types of cells, but indicated that the cytoplasmic mucoproteins differed from those of homologous mammalian cells. The protein: mucopolysaccharide ratio was lower and they appeared to be less sulfated.

Résumé

Des études cytologiques et cytochimiques sur des leucocytes basophiles et des mastocytes tissulaires de la salamandre aquatique (Diemyctylus viridescens) ont mis en évidence des différences morphologiques entre ces deux types de cellules. Les leucocytes basophiles contiennent des granulations plus grosses et moins de cytoplasme que

les mastocytes. Il ne fut pas possible de déceler par des méthodes cytochimiques [illegible] différences significatives des protéines et des mucopolysaccharides. Par contre l[illegible] démontrer que les mucoprotéines cytoplasmatiques diff[illegible] de ce[illegible] de [illegible] homologues des mammifères. Le report protéines/mucopolysaccharides [illegible] est plus [illegible] et leur contenu en souffre est moindre.

Zusammenfassung

Zytologische und zytochemische Untersuchungen an basophilen L[illegible] Gewebsmastzellen des Wassermolches (Diemyctylus viridescens) ergaben [illegible] sche Unterschiede zwischen den beiden Zelltypen. Basophile enthal[illegible] g[illegible] und [illegible] enger Zytoplasma als Mastzellen. Zytochemische U[illegible] tersuchun[illegible] und Mucopolysaccharide ergaben keine signifikanten U[illegible] terschiede, zeigten jed[illegible] die zytoplasmatischen Mucoproteine [illegible] on denjenigen entsprechender Säugetie[illegible] abweichen. Sie enthielten weniger Sulfatgruppen und das Protein-Mucopoly[illegible] Verhältnis [illegible] ar niedriger.

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Normierung der Hämoglobinometrie

Mitteilung der Hämometerprüfstelle

Beim 9. Kongreß der Europäischen Gesellschaft für Hämatologie in Lissabon wurde im Rahmen eines Symposions am 31. August 1963 eine Standardisierungs*kommission gegründet und ein Beschluß über die Standardisierung der Hämoglobinometrie* gefaßt. Im Gründungsprotokoll der Standardisierungskommission der Europäischen Gesellschaft für Hämatologie wird gefordert, daß in den einzelnen Ländern nationale (oder regionale) Kommissionen bzw. Prüfstellen einzurichten seien, die die Beschlüsse der Europäischen Kommission in ihren Ländern propagieren und deren Durchführung überwachen sollen. Die Deutsche Gesellschaft für Innere Medizin hat am 5. April d. J. in Wiesbaden den Beschluß gefaßt, daß diese Aufgabe von der Hämometerprüfstelle der Gesellschaft übernommen wird. Diesem Beschluß haben sich bereits die Deutsche Hämatologische Gesellschaft, die Deutsche Gesellschaft der Fachärzte für Laboratoriumsdiagnostik und die Deutsche Gesellschaft für Bluttransfusion angeschlossen. Ein Vorschlag für einen ähnlich lautenden Beschluß wird auch auf den nächsten Sitzungen der Gesellschaft für Klinische Chemie eingebracht werden. Ferner erhielt die Hämometerprüfstelle vom Bundesminister für Gesundheit einen Forschungsauftrag betreffend Standardisierung diagnostischer Methoden.

Auf Grund dieser Beschlüsse und Aufträge haben wir in Zusammenarbeit mit Herrn F. GASTER vom Fachnormenausschuß Feinmechanik und Optik einen Norm-Entwurf ausgearbeitet, wobei soweit als möglich, auf den Beschluß der Deutschen Gesellschaft für Innere Medizin (Acta haemat. *27*, 369, 1962) zurückgegriffen wurde. Der Norm-Entwurf enthält folgende Punkte: 1. Zweck, 2. Begriff Hämoglobin, 3. Maßeinheiten und Vergleichswert, 4. Standard-Lösung von Hämoglobin(cyanid), 5. Bestimmungsverfahren, 6. Photometer und sonstige Meßgeräte, 7. Eichkurven, 8. Durchführung. Der Norm-Entwurf enthält auch ausführliche Erläuterungen. *Der Norm-*Entwurf wird der Öffentlichkeit zur Stellungnahme vorgelegt. Einsprüche und Änderungsvorschläge können (in zweifacher Ausfertigung) *bis 31. Januar 1965* an den Fachnormenausschuß Feinmechanik und Optik eingereicht werden. Der Text des Norm-Entwurfes kann beim Beuth-Vertrieb (Berlin 15, Köln und Frankfurt/M) unter der Nummer DIN 58931 (7. 64) Bl. 1 bestellt werden (Preis DM 2.60). Es ist geplant, in einem weiteren Blatt dieser Norm noch die Anforderungen an Standard-Lösungen und deren Herstellung festzulegen. Darüber hinaus sollen weitere Normen über hämatologische Methoden erarbeitet werden. Über das Erscheinen der endgültigen Norm DIN 58931 sowie über das Erscheinen weiterer Norm-Blätter und internationaler Standardisierungsbeschlüsse wird auch in Zukunft an dieser Stelle berichtet werden.

K. G. von Boroviczény und N. K[illegible]

Acta haemat. 32: 257–264 (1964).

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Protein Metabolism and Erythropoiesis

III. The Erythroid Marrow in Protein-Starved Rats and Its Response to Erythropoietin

By KENJIRO ITO, JOHN W. SCHMAUS and KURT R. REISSMANN

An absence of protein in the diet severely and rapidly depresses erythropoiesis in the rat. No evidence was found in preceeding studies (1, 2) which would attribute this depression to a deficiency of protein precursors needed for the synthesis of red cells. The injection of erythropoietin markedly accelerated erythropoiesis in protein-starved rats and prevented the anemia which otherwise developed. After exposure to lowered barometric pressure, the plasma of protein-starved rats contained significantly less erythropoietin than the plasma of equally exposed rats receiving a complete diet. The depression of erythropoiesis during protein starvation was therefore attributed to a diminished formation of erythropoietin.

The arrest of erythropoiesis is nearly complete when young rats (100 g body weight) are placed on a protein-free diet. Their reticulocytes disappear from the blood within ten days, and their 24-hour radio-iron incorporation is reduced from the normal range of around 40% to a fraction of 1%. It is the purpose of this study to describe the morphology of the erythroid marrow in such rats, and to examine quantitative and temporal aspects of their erythroid response to the injection of erythropoietin.

Methods

Female Sprague Dawley rats weighing 90 ± 5 g were used in all experiments. Control rats were given Purina Rat Chow. Experimental rats received ad libitum Protein Depletion Diet (Nutritional Biochemical Corp.) containing 84% dextrin, 9% corn oil, 2% agar, 1% cod liver oil, and 4% vitamin-salt mixture. At the time indicated

Supported by U. S. Public Health Service Grant AM 07239-01

Table I

Erythroid cell counts (per 1000 marrow cells) in protein-starved rats (mean and standard error of mean 4 rats per group)

Dietary Day	Hemato-crit %	Reticulo-cytes %	Proerythro-blasts	Pronormo-blasts	Normoblasts early	Normoblasts late
Before	42	7.6	4±2	16±3	74±12	147±23
4	48	0.7	4±1	6±2	12±6	31±3
6	47	0.1	2±1	3±1	11±9	14±6
10	46	<0.1	1±1	0±1	1±1	4±2
17	46	<0.1	1±1	2±1	1±1	2±1
[illegible]6	41	0.3	2±2	4±2	5±3	6±3
[illegible]6	49	<0.1	1±1	2±1	1±0	2±1
35	33	0.9	3±1	3±1	18±6	21±7
35*	47	<0.1	0±1	0±2	2±2	2±1
Rat	63	0.3	2±2	7±2	11±5	[illegible]1±6
Mouse*	68	<0.1	1±1	1±1	2±1	3±2

* Received 0.5 ml red cells intravenously on day 20 and 30.
Normal diet, made polycythemic by hypertransfusion

In the tables, the rats were killed and the femur split lengthwise for removal of marrow. In one experimental series (fig. 1) marrow was obtained by needle biopsy from the left femur before and from the right femur after erythropoietin injection. The dry films were stained with Wright-Giemsa stain. One thousand bone marrow cells were counted in two different coverslide preparations of each marrow. Erythropoietin was obtained according to Borsook's method (3) from plasma of rabbits made rapidly anemic by bleeding plus injection of phenylhydrazine. The erythropoietic activity of the extract was calibrated against Erythropoietin Standard A (N.I.H. Bethesda and N.R.I. London). The nitrogen content of the extract equalled approximately 0.6 mg N per unit erythropoietin.

Cytological criteria for classification, in order of immaturity: Proerythroblasts, 20—30 μ diameter. Intensely blue cytoplasm, large nucleus with very fine chromatin reticulation and well defined nucleoli. Pronormoblasts, 15—20 μ, cytoplasm lighter in color, nucleus is coarser and darker. Early normoblasts, 7—12 μ, nucleus distinctly pyknotic but cytoplasm shows no polychromasia. (Rat normoblasts retain basophilia much longer than corresponding cells in other species (4) and identification is mainly based on characteristic chromatin pattern of nucleus.) Late normoblasts, 7 μ, nucleus very pyknotic, cytoplasm poly- or orthochromatic.

Results

The effect of protein deprivation on hematocrit, blood reticulocytes, and erythroid marrow composition is shown in table I. After 10 days the marrow was nearly void of erythroid elements, but the few remaining cells were normal in appearance. Longer duration of protein starvation increased rather than decreased the cellularity of erythroid marrow. This was very likely the result of a resumption of erythropoietin formation in response to the progressive anemia. Two groups of protein-starved rats received small red cell infusions

Table II

Response to single injection of 2.4 units erythropoietin given on eleventh day of protein starvation (mean and standard error of mean, 4 rats per group).

Day after injection	Hematocrit %	Proerythroblasts	Pronormoblasts	Normoblasts Early	Normoblasts Late	Blood Reticulocytes ‰
		Per 1000 Bone Marrow Cells				
0	45±1	1±1	0±1	1±1	3±2	0.1±0.04
0.5	46±2	12±3	2±2	2±1	4±3	0.2±0.01
1	47±1	14±2	32±6	36±10	8±4	0.4±0.06
2	47±1	12±1	38±5	157±17	55±16	6.0±1.5
3	45±2	4±3	18±5	87±11	131±27	26.8±5.1
4	43±1	3±2	4±6	10±17	89±11	46.7±4.3
5	45±1	1±1	2±2	6±9	36±18	14.6±9.8
7	47±2	2±1	3±1	1±1	7±4	0.7±0.4
9	43±4	3±1	2±2	2±2	8±2	0.5±0.1

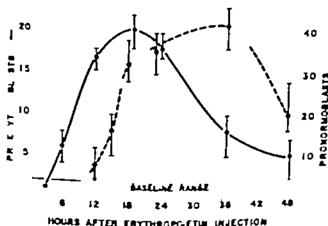


Fig. 1. Proerythroblasts and pronormoblasts (per 1000 marrow cells) in protein-starved rats after injection of 4 units erythropoietin. Pre-injection counts indicated as baseline range (3 rats per group)

to prevent the anemia and their marrow remained void of erythroid elements over an observation period of five weeks. Throughout this time the animals remained in good general condition and no abnormal cells were seen in their marrows. The erythroid differential counts of rats and mice, made polycythemic by hypertransfusion, are presented for comparison at the bottom of table I.

The period of maximal erythroid depression (11 to 18 days) was chosen to study the marrow response to a single subcutaneous injection of 2.4 units of erythropoietin (table II). All rats had reti-

preted as the entry of cells from an unlabeled progenitor compartment. The rate of influx varied with erythropoietic stimulation. Since the latter is regulated by erythropoietin, it was logical to infer that the hormone induces stem-cell differentiation, perhaps by induction of enzymes which are characteristic of the erythroid cell (11 12)

The decrease in proerythroblasts after erythropoietin suppression and their re appearance after its injection have been cited in support of this concept, although the same results were to be expected if erythropoietin would accelerate the hemi homoplastic multiplication of proerythroblasts. A complete disappearance of proerythroblasts can, in our experience not be achieved by any method of erythropoietin suppression. After several weeks of protein starvation for instance a few cells still remained which at least resembled proerythroblasts and which could have multiplied under the accelerating effect of erythropoietin. The quantitative data presented in fig 1 however render such a possibility highly improbable. The temporal relationship between pronormoblast and proerythroblast curves and their respective amplitudes signify a division of the former after 9 hours. The proerythroblast cell cycle in the rat thus seems to be somewhat shorter than the 13 hour cycle found in the dog (13) The mean proerythroblast count increased from 1 to 17 within 12 hours after intravenous erythropoietin injection. According to a hemi homoplastic multiplication, this would have required some 16 successive cell divisions with less than one hour between divisions. Generation times of such short duration are highly improbable and no cytologic evidence of mitotic activity was found. By exclusion, therefore, our findings support stem-cell differentiation as the source of the marked increase of proerythroblasts after erythropoietin injection. The excellent correlation between dose of erythropoietin and proerythroblast count (fig 2) furthermore proves that erythropoietin is not only a prerequisite of stem-cell differentiation but clearly exercises a rate-regulating function.

Summary

Omission of protein from the food of rats resulted within 10 days in nearly complete disappearance of erythroid marrow elements. A single injection of erythropoietin generated a wave of erythroid proliferation which commenced with an increase in proerythroblasts, progressed in orderly sequence through the erythron, and terminated with the release of reticulocytes. The proerythroblast count rose steeply

within 18 hours after intravenous injection of erythropoietin. On the basis of quantitative considerations, it is concluded that these proerythroblasts developed through differentiation of progenitor (stem) cells. Most, if not all, proerythroblasts divided into pronormoblasts after a cell cycle of 9 hours. A nearly linear relationship was found between logarithm of the dose of erythropoietin and percentage of proerythroblasts present 18 hours after its injection.

Résumé

Une nourriture sans protéine provoqua chez des rats une disparition presque totale des cellules de l'érythropoïèse dans la moelle osseuse. Une injection unique d'érythropoïétine amena une prolifération passagère de l'érythropoïèse qui commença par une augmentation des proérythroblastes, puis continua dans l'ordre par les cellules de l'érythron et se termina par la libération de réticulocytes. Le nombre des érythroblastes augmenta graduellement pendant 18 heures après l'injection d'érythropoïétine. Des considérations d'ordre quantitatif font penser que ces proérythroblastes se développent par différentiation à partir de cellules-souches. La plupart, si ce n'est tous les proérythroblastes, se divisèrent en pronormoblastes après un cycle cellulaire de neuf heures. La relation entre le logarithme de la dose d'érythropoïétine et le pourcentage de proérythroblastes se trouve être 18 heures après l'injection de celle-ci à peu près linéaire.

Zusammenfassung

Bei Ratten führte proteinfreie Ernährung innerhalb von 10 Tagen zu einem nahezu vollständigen Schwund der erythropoetischen Knochenmarkzellen. Eine einzelne Injektion von Erythropoetin erzeugte einen begrenzten Schub einer erythropoetischen Proliferation, der mit einem Anstieg der Proerythroblasten begann und über die normale Reihe des Erythrons verlief, um mit der Freisetzung von Reticulocyten zu enden. Die Zahl der Proerythroblasten stieg innert 18 Stunden nach intravenöser Injektion von Erythropoietin stufenweise an. Aus quantitativen Überlegungen wird geschlossen, dass diese Proerythroblasten sich durch Differenzierung aus Vorstufen (Stammzellen) entwickelten. Die meisten, wenn nicht alle Proerythroblasten gingen durch Teilung nach einem Zyklus von 9 Stunden in Pronormoblasten über. Es fand sich eine annähernd lineare Beziehung zwischen dem Logarithmus der Erythropoietindosis und dem Prozentwert der Proerythroblasten 18 Stunden nach der Injektion.

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Acta haemat. 32: 265–270 (1964)

From the Department of Medicine, University of Pavia (Head: Prof. P. Introzzi)

Formation of Tryptophan pyrrolase in Leukaemic Cells Incubated with RNA Obtained from the Spleen of Calf Embryo

By Sergio Esposito

Over the last few years various writers have demonstrated the possibility of affecting the evolution of certain tissues by incubating them in a culture containing RNA (1–3, 10). It has been observed, for example, that when an embryonic tissue is cultivated with the addition of RNA extracted from different organs, its own development is orientated towards forms similar to those of the tissue which originally furnished the RNA (6, 9, 12).

This phenomenon is also observed in cells of experimentally induced tumors in animals. It has been demonstrated in tumor cells that RNA obtained from liver can cause the formation of protein and enzymes of the type found in hepatic tissue, as, for example, albumin, tryptophan-pyrrolase and glucose-6-phosphatase (11, 15).

During the past year we have also tried to reproduce these phenomena in the cells of human neoplasias. The problem of obtaining a homogeneous cell population for incubation with RNA was resolved by using cells of the bone marrow from subjects affected with histioleukaemia. It often happens in these cases that the cell population consists almost entirely of mutually identical elements, which fact facilitates to a certain degree the demonstration of metabolical or morphological modifications.

In order to affect the evolution of these cells we proceeded to the extraction of RNA from the spleen of calf embryo. The extraction was effected during the period of erythropoietic and granulopoietic activity. Thus, a part of the RNA extracted should, theoretically speaking, contain the "information" required to modify the evolution of leukaemic cells. In this way it was possible to demonstrate that leukaemic cells, incubated with RNA, modify their structure

and assume forms similar under some ultrastructural aspects, to normal medullary cells (4)

De Carvalho has demonstrated lately that by injecting into the medullary cavity of leukaemic subjects RNA obtained from the haemopoietic cells of normal subjects, an improvement is achieved locally in the leukaemic picture (2) On the basis of these results, the American writer asked himself whether it was due to a simple non-specific haemopoietic stimulation or to an actual transformation of the leukaemic cells. The results, we obtained from cultures "in vitro (4 5) speak in favour of the second hypothesis. We had to determine if our results were due to a metabolic modification of the cells, or if they were produced by the culture mixture *in which the leukaemic cells were placed. In other words, it was* possible that the structural and functional changes taking place in the leukaemic cells were being conditioned by the continual passing of RNA from the culture mixture to the cells.

In order to throw light on this problem we set ourselves to study the metabolic behaviour of the leukaemic cells cultivated with RNA after the addition of a substance capable of blocking the endocellular production of RNA. In this connection we may remind ourselves that there are several substances, of which the best known is actinomycin, which are capable of inhibiting the production of RNA through the action of DNA (13) Of these various substances we selected 5-azauridine, on account of the fact that its action can be immediately checked by uridine. The use of these substances has previously demonstrated that the metabolic modifications in tumor cells of animals are not due to the continued action of RNA but to the biosynthesis of RNA in the cells themselves (11)

In order to study the possibility of transferring to leukaemic cells the information" contained in the splenic tissue of the embryo we chose the determining factor of tryptophan-pyrrolase. This enzyme has been shown to be absent in the cells used in these experiments, while it is present in the spleen of calf embryo.

Materials and Methods

Characteristics of the cells incubated. The cells used for the experiment were obtained from case of histioleukaemia. The cell population consisted of elements which were 94 mutually identical. Their examination under the electronic microscope showed that no ergastoplasmic organization at all was present. Nor was any tryptophan-pyrrolase activity revealed.

RNA used in the experiments. The RNA was obtained by Kirby method (7) from the spleens of embryo calves. The splenic tissue was in state of erythropoietic and granulopoietic activity It was also shown to contain tryptophan-pyrrolase.

Culture technique. The material required for the cultures was obtained by sternal puncture into syringe containing traces of heparine. A few ml of Tyrode solution were added and the whole was then placed in an ice-box at 1 °C. After about one hour the medullary matter that had risen to the surface was removed and re-suspended in culture mixture consisting of 8 parts basal medium and 2 parts old serum of horse By means of gentle agitation the particles were dissolved. The cells were then counted. After the addition of specific quantity of culture mixture the final concentration was brought to 10,000 cells/mm³

The cellular suspension was then divided and placed in series of matrasses. T some of these matrasses was added 0.5 mg/ml of RNA obtained from the spleen of calf embryo. The remaining matrasses served as controls. All matrasses were transferred to metabolic agitator The period of incubation was protracted, under sterile conditions, for 12 hours at 37 °C.

At the end of the incubation period the tryptophan-pyrrolase was determined by Knox and Mehler's method (8). The effect of the 5-azauridine on the production of tryptophan-pyrrolase was determined according to the method described by Neo et al. (11). The 5-azauridine (15 mg/ml) was added after lapse of 1 ½ hours from the beginning of the experiment, the uridine (20 mg/ml) after 2 ½ hours.

Results

The results obtained are shown in figs. 1 and 2. These diagrams give the calculated averages of 10 different cultures for each experiment (culture of leukaemic cells, culture of leukaemic cells + RNA, culture of leukaemic cells + RNA + 5-azauridine + uridine)

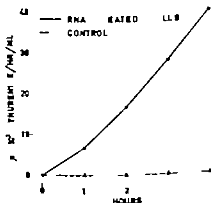


Fig. 1. Formation of kynurenine caused by tryptophan-pyrrolase in untreated leukaemic cells (control) and in leukaemic cells incubated with RNA obtained from the spleen of calf embryo.

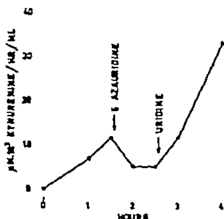


Fig. 2 Behaviour of the kynurenine formation in leukaemic cells incubated with RNA, following the addition of 5-azauridine or uridine.

Fig 1 shows that the leukaemic cells used in these experiments are themselves not capable of producing kynurenine In the cells cultivated with RNA on the other hand, the formation of kynurenine is very marked.

The presence of 5-azauridine has effected an immediate decrease in the production of kynurenine by the cells cultivated with RNA (fig 2) Immediately following the blocking of the action of 5-azauridine by means of uridine, the production is resumed.

Discussion

The fact that no kynurenine was produced demonstrates the absence of tryptophan-pyrrolase in the leukaemic cells used in the experiment. Let us remember that we were dealing with a stock of highly undifferentiated cells, possessing no ergastoplasmatic organization and therefore almost no metabolic activity For this reason they were particularly well-adapted for the attempt at transmitting metabolic characteristics from another tissue by means of incubation with RNA obtained from the latter

The formation of kynurenine demonstrated the appearance of tryptophan pyrrolase in the leukaemic cells cultivated with RNA. This result proves that even in leukaemic cells it is possible to cause the appearance of a specific enzyme as has been shown in other experiments with normal haemopoietic cells (14)

The important fact to underline is that the addition of 5-azauridine caused an immediate cessation of the formation of kynurenine. As azauridine inhibits the synthesis of RNA by means of DNA, it must be conceded that the metabolic modifications observed depended on the production of RNA on the part of the DNA in the incubated cells and not on the continual passage of the substance from the culture mixture to the cells. The connexion between the phenomena is demonstrated also by the inhibiting effect of uridine on 5-azauridine with immediate resumption of the formation of kynurenine. In conclusion the RNA present in the culture mixture, by entering into the leukaemic cells is capable of initiating the spontaneous production of RNA on the part of the cells themselves. This obviously forces us to admit the existence of a direct influence on the DNA level.

Summary

In the leukaemic cells cultivated with RNA obtained from the spleen of calf embryo, the appearance of tryptophan-pyrrolase has been demonstrated. This enzyme has not been observed in the cell stock used for the experiment, while it is present in the splenic tissue of the calf embryo. The addition of 5-azauridine and uridine to the culture mixture showed the appearance of the enzyme in the leukaemic cells to be due to direct influence on the DNA of the cells themselves.

Résumé

La formation de pyrrolase de tryptophane a pu être démontrée dans des cellules leucémiques cultivées à l'aide d'ARN obtenu de la rate de veau. Cet enzyme ne se trouve pas dans les cellules employées pour cette expérience, mais cependant dans le tissu splénique de l'embryon de veau. L'addition d' 5-azauridine et d'uridine au milieu culturel démontra que l'apparition de cet enzyme dans les cellules leucémiques est due à une influence directe sur l'ADN de ces cellules.

Zusammenfassung

In leukämischen Zellen, die mit RNA aus embryonaler Kalbsmilz kultiviert wurden, konnte die Bildung von Tryptophan-Pyrrolase nachgewiesen werden. Dieses Enzym findet sich nicht in dem für die Versuche verwendeten Zellmaterial, während es im Milzgewebe von Kalbsembryonen vorhanden ist. Die Zugabe von 5-Azauridin und Uridin zum Kulturmedium zeigte, daß das Auftreten des Enzymes in leukämischen Zellen durch eine direkte Beeinflussung der DNA dieser Zellen selbst bedingt ist.

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Acta haemat. 32: 271–279 (1964).

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RNA and Protein Metabolism in Normal Human Erythroblasts and Granuloblasts*

By U. Torelli, G. Grossi, T. Artusi, G. Emilia, I. R. Attiya** and C. Mauri

It is well known today that several different types of RNA exist in the cell and play a different role in the execution of the message held in DNA. These types differ in many respects, such as base composition, molecular size, synthetic pathways and turnover rate (18). As far as the latter is concerned, for example, we are aware of (11, 19) the high metabolic rate of the so-called "messenger" RNA, i.e. the fraction involved in the transfer of the genetic information from DNA to ribosomal particles. Microsomal RNA is on the other hand metabolically stable (4).

The knowledge of the development of the various RNAs in the bone marrow cells at different stages of maturation should be of great interest for a better metabolic and functional characterization of these cells. The measurement of intracellular RNA turnover is very difficult, owing to the existence of a large intracellular precursor pool which is balanced with radioactive exogenous [illegible] very slowly. However, the study of the RNA turnover rate at cellular level by the autoradiographic method has been greatly facilitated today by the discovery of the properties of actinomycin [illegible]. As a matter of fact this substance, even at low concentrations [illegible] pletely inhibits the synthesis of all the RNA fractions [illegible] itself to DNA, so that the entire amount of the [illegible] appears to be synthesized in a DNA-dependent reaction [illegible]. Since actinomycin seems to have no effect on the [illegible] phosphorylase like enzyme which degrades nucleic [illegible] appears possible today to investigate the intracellular [illegible]

* Research aided by grant from the Consiglio Nazionale [illegible]
** International Atomic Energy Agency Fellow

of the labelled RNA in conditions in which simultaneous synthesis of RNA is completely prevented.

We have therefore thought that the study of the RNA turnover rate in erythroblastic and granuloblastic cells by autoradiography might allow some insight into the proportion in which RNAs with different turnover rates coexist therein. Of course it cannot be excluded that some artifacts may be produced by treating the cells with actinomycin. However, as it was pointed out by REICH (16), it appears possible that actinomycin may be completely innocuous to all cellular processes not primarily involved in DNA metabolism. This paper therefore deals with the autoradiographic study of the uptake of tritiated precursors of RNA in erythroblasts and granuloblasts and of their release rate after treatment with actinomycin. At the same time the uptake of tritiated leucine was studied, owing to the different functional meaning of the various RNA fractions in protein synthesis.

Materials and Methods

H^3-cytidine and H^3-uridine were employed for the study of RNA metabolism, and H^3-leucine for the study of protein synthesis. It must be observed that the multiplying bone marrow cells synthesize also DNA, and cytidine and uridine may be incorporated into both DNA and RNA. However, since DNA is synthesized only during a certain phase of the intermitotic cycle (10), cells which are not in phase of DNA synthesis incorporate cytidine and uridine only into RNA. It is therefore possible to measure the turnover of RNA by the incorporation and release of labelled nucleosides by selecting cells which are not in phase of DNA synthesis. This aim can be achieved, as was pointed out by HAMPL (7), by adding H^3-thymidine to the medium. Cells in phase of DNA synthesis incorporate H^3-uridine or H^3-cytidine into both RNA and DNA, and H^3-thymidine into DNA. The nuclei of such cells are therefore much more heavily labelled than the nuclei of cells not in phase of DNA synthesis, and the two groups of cells are easily distinguished. All the radioactive substances used were obtained from Radiochemical Centre, Amersham, Bucks.

For the study of RNA metabolism, 28 ml of marrow blood, aspirated from the posterior iliac spine, were distributed in seven centrifuge tubes where 1 ml of Gey solution had been added, containing 50 μc of H^3-cytidine and 5 μc of H^3-thymidine so that the final concentration was of 10 μc/ml and 1 μc/ml respectively. The first tube was left at 37° C for 55 min, and at the end of this period was centrifuged at 2000 rpm for 5 min. The leucocyte layer was aspirated with a Pasteur pipette, resuspended in few drops of plasma and smeared immediately on slides. 60 min after the beginning of the experiment, 40 μg of actinomycin D were added to three tubes, and leucocyte smears of treated and untreated bone marrow cells were obtained after 30, 60 and 120 min of incubation.

The experiment was repeated, under as similar conditions as possible, using H^3-uridine. Three normal bone marrows were examined with the above technique. During further experiments on myeloid cells of the same subjects, in order to differentiate DNA from RNA synthesis, instead of adding H^3-thymidine to the medium, nuclear [illegible]

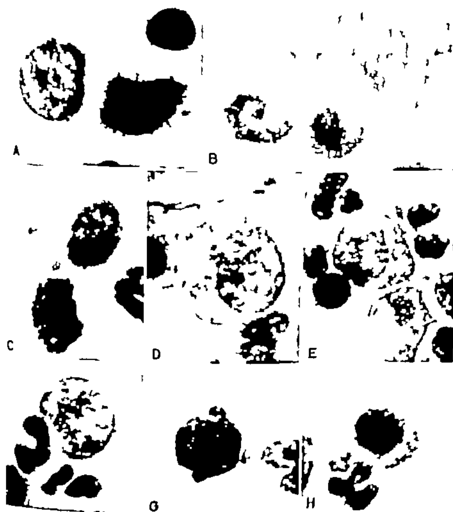


Fig. 1. Labelled cells after in vitro incubation of bone marrow with H^3-thymidine and H^3-cytidine, or H^3-leucine. A. Myeloblast labelled with H^3-[illegible] [illegible] labelled with H^3-cytidine. [illegible] B. Promyelocyte [illegible] labelled with H^3-uridine. [illegible] H^3-thymidine and myelocyte [illegible] labelled with [illegible] thymidine. D. Promyelocyte labelled with [illegible] Myeloblast labelled with H^3-leucine. F. Proerythroblast labelled with [illegible] labelled with H^3-uridine. H. Polychromatic erythroblast labelled with [illegible]

was employed. After fixation in Carnoy [illegible] fluid the slides were immersed for [illegible] hours in [illegible] solution containing 0.1 mg of deoxyribonuclease (Sigma) per ml of Gomori [illegible] maleate (5) buffer with $MgSO_4$ [illegible] H_2O 0.2 M.

To study protein [illegible]ynthesis, bone marrow samples were incubated [illegible] H^3 leucine at the concentration of 10 μc/ml, and cell smears obtained after 60 min.

The smears, fixed in Carnoy [illegible] fluid, were covered with Kodak A.R. 10 stripping film and maintained in [illegible] refrigerator at +2 °C in dry air with silica gel as [illegible] desiccant. After exposure times adjusted to give grain densities suitable for counting, the autoradiographic preparations were developed, fixed, washed and stained [illegible] M.G.G.

The study of [illegible] fairly large number of the more immature elements was difficult when H^3-thymidine was added to the tubes, because many of these cells appeared in phase of DNA synthesis. However at least 40 elements of each type were examined on each slide for myeloblasts, promyelocytes, proerythroblasts and basophilic erythroblasts, and 100 elements for the other cell types. The number of grains was counted [illegible]. The standard error of the mean grain counts was seldom higher than 15% of the mean.

As far as the effect of actinomycin is concerned, preliminary experiments showed that actinomycin, added to the bone marrow cultures few minutes before labelled nucleosides, completely inhibited the uptake of the two precursors. It can therefore be assumed that nucleoside uptake in our experimental conditions was [illegible] reliable index of RNA synthesis. Terminal addition could account not even for [illegible] part of the radioactivity observed in the cells.

Table 1

RNA and protein radioactivity in normal bone marrow cells after 1 hour incubation with H^3-cytidine, H^3-uridine and H^3-leucine. The figures indicate the mean grain counts and standard errors obtained in a single experiment.

Cell type	Cytidine		Uridine		Leucine		Cytidine/leucine ratio	Uridine/leucine ratio
Myeloblasts	86.7 ±	15.7	79.3 ±	11.0	61.8 ±	7.6	1.40	1.30
Neutro. promyel.	77.4 ±	10.4	70.1	6.1	93.1	7.9	0.80	0.75
Neutro. myeloc.	38.8 ±	[illegible]	31.5	[illegible]	[illegible]8.1	8.7	0.6[illegible]	[illegible]
Neutro. metamyel.	10.2	2.7	12.2	1.4	49.7	6.6	0.20	[illegible]
Proerythroblasts	78.6	13.5	62.7	12.7	93.4	[illegible]	0.83	[illegible]
Basoph. erythr.	59.5 ±	13.7	45.1	9.5	86.3	9.7	0.63	[illegible]
Polychr. erythr.	29.7	1.5	18.7 ±	2.0	56.1	11.33	0.50	[illegible]
Orthochrom. erythr.	7.5	1.6	5.6	0.8	27.1	4.2	0.27	[illegible]

Results

Uptake of precursors into RNA and protein. After incubation with tritiated nucleosides, the grains appeared only over the nucleus: the labelling with tritiated leucine on the contrary appeared both over the nucleus and the cytoplasm, but the number of grains over the cytoplasm was often much higher than over the nucleus (fig. 1). However the labelling values were always referred to the cell as a whole. The uptake rate of labelled nucleosides was always higher in the early forms than in mature ones. The same thing occurred when leucine was used, except in myeloblasts, where the labelin[illegible]

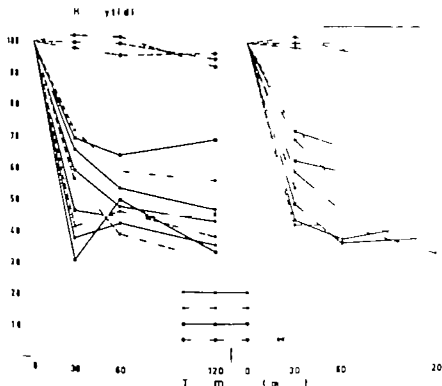


Fig 2. Effect of actinomycin D on RNA radioactivity in normal human erythroblasts. The percentages of the average grain counts for each cell type are plotted against the interval after the addition of the actinomycin to the medium.

was lower than in promyelocytes. The decrease of the uptake as maturation went on was always greater with nucleosides than with leucine, so that the nucleoside/aminoacid ratio in the immature forms was considerably higher than in mature ones (table I)

Effect of the inhibition of RNA synthesis by actinomycin D While average labelling steadily increased throughout the incubation period in all types of cells, though at variable rate, after adding actinomycin to the tubes the average grain counts showed a sharp decrease in the immature forms of both the erythroblastic (fig 2) and granuloblastic (fig 3) series. On the contrary in metamyelocytes and in orthochromatic erythroblasts the labelling did not show a significant decrease during the incubation with actinomycin. No significant difference was found in the results obtained with the two nucleosides.

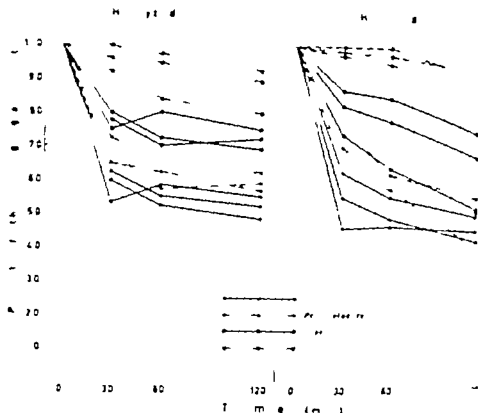


Fig. 3 Effect of actinomycin on RNA radioactivity in normal human granulocytes

Discussion

Two main findings are the result of our investigation first, it was observed that for every molecule of leucine taken up into protein, immature myeloid cells synthesize a much greater amount of RNA than mature forms do. Secondly it was observed that most of the RNA synthesized in immature myeloid cells is rapidly degraded.

A short lived RNA was repeatedly observed in mammalian cells (9, 12, 18) but its functional meaning is far from being clear. As a matter of fact a rapid metabolic rate is one of the main features of the microbial messenger RNA (11). However the observed stability of the template in some protein producing animal cells (9, 14) led some authors to postulate that the regulation of protein synthesis in animal cells is not effected by a flow of unstable RNA molecules from the DNA.

The demonstration of the function of pol r mes) in animal cells (6) and the isolation of ... from liver cell cytoplasm (13) and from pu... seem to confirm that the mechanism by which ... is translated to ribosomes is basically similar ... cells.

As far as the relationship between unstable ... synthesis in animal cells is concerned, it must b... ALLFREY (1) observed that the uptake of amino-a... requires the prior synthesis of messenger RNA. H... observe that after inhibition of messenger synthes... the protein synthesis rate remains normal for ab... HARRIS (9) recently concluded that most of the unstable ... animal cells may be destroyed without influencing directly th... protein synthesis rate.

It seems possible to postulate that the rapidly degraded RNA fraction observed in immature granuloblastic and erythroblastic cells may be considered as a messenger fraction. If it is so the lack of unstable RNA in mature myeloid precursors and the stability of RNA in haemoglobin producing reticulocytes (14) seem to indicate that the information for protein synthesis is elaborated only during the phase of active proliferation of the immature cells, so that presumably any messenger RNA is attached to the ribosomes during the early stages of the functional maturation, in agreement with the observations of ARNSTEIN et al. (2) In this connection it must be remembered that in both chicken nucleated erythrocytes (3) and rat reticulocytes (5) the autoradiographic study of the uptake of tritiated precursors of RNA and protein showed that RNA synthesis stops before protein synthesis.

Summary

RNA and protein synthesis was studied in normal human myeloid cells by measuring autoradiographically the uptake of tritiated nucleosides and leucine. The rate of RNA breakdown in these cells was studied by evaluating the release of the label after treatment with actinomycin. For every molecule of leucine taken up into protein, immature myeloid cells synthesize much greater amount of RNA than mature forms. It was also observed that in immature myeloid cells most of the RNA undergoes rapid breakdown.

The authors postulate that the RNA fraction with high turnover rate synthesized in immature granuloblasts and erythroblasts may be considered as messenger fraction. Since an unstable RNA fraction is not evidenced in mature cells (metamyelocytes and orthochromatic erythroblasts) it seems possible that the information for protein synthesis

is laborated during the proliferation phase of the immature cells, so that any messenger RNA is attached to the ribosomes during the early stages of maturation.

Résumé

La synthèse de l'ARN et des protéines a été étudiée dans des cellules médullaires normales par analyse autoradiographique de l'absorption de nucléosides et de leucine marqués au tritium. La vitesse de désintégration de l'ARN dans des cellules a été déterminée en mesurant la diminution du marquage après un traitement à l'actinomycine. Pour chaque molécule de leucine inclue dans la formation des protéines les cellules médullaires immatures synthétisent une quantité bien plus grande d'ARN que les cellules mures. Il fut en outre possible de démontrer que dans les cellules médullaires immatures la plus grande partie de l'ARN est très rapidement dégradée.

Les auteurs postulent que la fraction d'ARN ayant un turnover rapide et synthétisée dans les granuloblastes et les érythroblastes immatures soit considérée comme une fraction messagère. Comme il ne se trouve pas de fraction de l'ARN instable dans les cellules mures (métamyélocytes et érythroblastes orthochromatiques) il semble possible que les informations nécessaires à la synthèse des protéines soient élaborées pendant la phase de prolifération des cellules immatures de telle façon que chaque ARN messager soit fixé aux ribosomes durant les premiers stades de la maturation.

Zusammenfassung

An normalen menschlichen Knochenmarkzellen wurde die Synthese von RNA und Protein untersucht durch autoradiographische Bestimmung der Aufnahme von mit Tritium markierten Nukleosiden und Leucin. Die Abbaurate von RNA in diesen Zellen wurde untersucht, indem der Schwund der Markierung nach Behandlung mit Actinomycin bestimmt wurde. Für jedes in Protein eingebaute Molekül Leucin synthetisieren unreife myeloische Zellen eine wesentlich grössere Menge von RNA als reife. Es konnte ferner gezeigt werden, daß der Großteil der RNA in unreifen myeloischen Zellen rasch abgebaut wird.

Es wird postuliert, daß die rasch umgesetzte RNA-Fraktion, die von unreifen Granuloblasten und Erythroblasten synthetisiert wird, als Überträgerfraktion zu betrachten sei. Da eine unstabile RNA-Fraktion in reifen Zellen (Metamyelocyten und orthochromatische Erythroblasten) nicht vorkommt, erscheint es möglich, daß die Information für die Proteinsynthese während der Proliferationsphase unreifer Zellen geliefert wird, wobei jede Überträger-RNA während eines frühen Reifungsstadiums an die Ribosomen gebunden wird.

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Acta haemat. 32: 280–291 (1964)

Aus der Universitäts-Kinderklinik, Tübingen (Direktor: Prof. Dr. K. Be[illegible])

Die Bedeutung der γ-Ketten für die Eigenschaften des fetalen Hämoglobins

Von Enno Kleihauer und Nikolaus Miltényi*

Zahlreiche Untersuchungen über das physikalisch-chemische und funktionelle Verhalten des fetalen Hämoglobins (HbF) haben gezeigt, daß dieser normalerweise nur in der Fetalzeit und bis zum Ende des ersten Lebenshalbjahres vorkommende Blutfarbstoff in vieler Hinsicht mehr vom bleibenden Blutfarbstoff (HbA) differiert als manches anomale Hämoglobin. Das wird verständlich durch die Unterschiede in der strukturellen Zusammensetzung des Globinanteils, der für die Spezifität des Hämoglobins verantwortlich ist.

Alle normalen humanen Hämoglobine enthalten zwei α-Ketten, während das zweite Kettenpaar – die β-, γ- oder δ-Kette – infolge der unterschiedlichen Sequenz der Aminosäuren dem Blutfarbstoff die charakteristischen Merkmale verleiht (1–8). Nach der Art der Kettenanordnung erhält der Hauptanteil des adulten humanen Hämoglobins (HbA₁) das Strukturymbol $\alpha_2\beta_2$, die elektrophoretisch langsamer wandernde Minorfraktion HbA_2 das Symbol $\alpha_2\delta_2$ und das fetale Hämoglobin das Symbol $\alpha_2\gamma_2$. Die anomalen Hämoglobine sind im Prinzip wie das HbA gebaut, wobei allerdings entweder in der α- oder β-Kette eine Aminosäure gegen eine andere ausgetauscht ist. Ausnahmen bilden das HbH, das nur aus β-Ketten (β_4) besteht, und das Hb Barts, das nur γ-Ketten (γ_4) enthält. Beim Hb-Lepore hat dagegen ein crossing over zwischen der β- und δ-Kette statt gefunden (9).

Die bekanntesten Unterschiede zwischen HbF und HbA finden sich in dem Verhalten gegenüber Alkali (10–13), Säure (14–15) und Hitzeeinwirkung (16), in der elektrophoretischen Wanderungsgeschwindigkeit (17), der spektralen Absorption im UV-Bereich (18), der Antigeneigenschaft (19) und der Oxydation durch Kaliumferricyanid und Natriumnitrit (20, 21).

Da die α-Ketten von HbA und HbF identisch sind (2, 7, [illegible]), müssen die differenten Eigenschaften beider Hämoglobine durch die β- bzw. γ-Kette bedingt sein. Der Beweis dafür konnte einfach erbracht werden, wenn sich die beiden Ketten in intaktem Zustand

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solieren ließen. Da das in optimaler Wei H
α-Ketten möglich ist (23) muß man den Un
dissoziierung wählen (24) Das Prinzip der Hybr
folgendes Bringt man eine Hämoglobinlösun in
so dissoziieren die Halbmoleküle des Hämo o
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Milieu neutralisiert, so treten sie wieder ... ination) $\alpha_2+\beta_2 \rightarrow \alpha_2\beta_2$. Erfolgen Dissoziation ui
mit zwei differenten Hämoglobinen so bildet
freien Austauschbarkeit der Halbmoleküle neben
gangsfarbstoffen zwei neue Hämoglobine (H Li
tionen lassen sich im Stärkeblock elektrophoretisch
isolieren.

Methoden

Die *Hybridisierung* wurde in Anlehnung an das V ... on H ... et al (25) durchgeführt. Mit Tetrachlorkohlenstoff gereinigte und mit Kohlenmonoxyd ersetzte Hämolysate (6–8 g%) aus Erwachsenen- und Nabelschnurblut wurden je mit gleichen Teilen eines Hämolysates aus Hundeblut (Hbcan) und / Volumen 0,5 m Acetatpuffer pH 4,7 gemischt. Diese Mischungen wurden in Cellophanmembranen 24 Stunden im Kühlschrank gegen den gleichen Acetatpuffer (Dissoziationsphase) und weitere 24 Stunden gegen einen Tris-EDTA-Borat-Puffer pH 9,0 (Rekombinationsphase) dialysiert. Anschließend wurden die Proben zur Entfernung denaturierten Hämoglobins 10 Minuten bei 6000 U./min zentrifugiert, erneut mit Kohlenmonoxyd ersetzt und elektrophoretisch getrennt.

Die *Trennung der Hybride* erfolgte im Stärkeblock, Veronal-Veronal-Na-Puffer pH 8,6, 400–500 Volt, 60–80 mAmp. über 16–20 Stunden. Aufgrund der unterschiedlichen Wanderungsgeschwindigkeiten stellen sich die Fraktionen von der Anode zur Kathode in der folgenden Anordnung dar: $\alpha_2^{can}\beta^A$ — $\alpha_2^{can}\gamma^F$ — $^A\beta$ (HbA) — $\alpha^F\gamma^F$ (HbF) + $\alpha_2^{can}\beta^{can}$ (Hbcan) — $\alpha\ \beta^{can}$ + $\alpha^F\beta^{can}$ (Abb. 1) Die einzelnen Fraktionen sind im allgemeinen so scharf getrennt, daß sie sich relativ sauber isolieren lassen. Lediglich $\alpha_2^{can}\beta^A$ und $\alpha_2^{can}\gamma^F$ liegen ziemlich dicht beieinander so daß die jeweiligen Eluate eine geringe Zumischung des andern Hybrids enthalten können. HbF und Hbcan laufen als einheitlich breite Komponente, deren Isolierung dadurch nur schlechter gelingt. Die elektrophoretisch getrennten Hybride wurden mit der Stärke ausgeschnitten, mit Aqua bidest. verrührt und durch scharfes Zentrifugieren aus der Stärke eluiert. Nach 24stündiger Dialyse gegen Aqua bidest. im Kühlschrank wurden die Proben vor den jeweiligen Versuchen nochmals scharf zentrifugiert.

Die *Spektralkurven* wurden nach Zusatz von /₁₀ Volumen eines 2 m Phosphatpuffers, pH 6,8 in Quarzküvetten mit 1 cm Schichtdicke im automatisch registrierenden Zeiß-Spektralphotometer im UV-Bereich zwischen 325 und 270 nm geschrieben.

Die *Alkalidenaturierung* erfolgte im optischen Test nach Jonxis und Visser (17) bei einer Wellenlänge von 630 nm. Die Eluate wurden zu diesem Zweck nach der 24stündigen Dialyse nochmals gegen K_3FeCN und anschließend gegen KCN dialysiert, um den gesamten Farbstoff in Cyanhämiglobin zu überführen.

Hier sowie in Abb. 3–7 wird zwischen α_1 und α_2^F unterschieden. Dies soll nur die Herkunft der jeweiligen α-Ketten anzeigen selbstverständlich sind die beiden α-Ketten identisch.

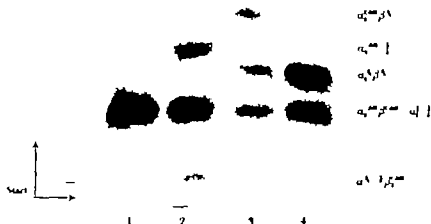


Abb. 1 Elektrophoretische Trennung der Hybride und Ausgangshämolysate 1 Nabelschnur und Hundehämoglobin nicht hybridisiert; 2. Nabelschnur und Hundehämoglobin nach Hybridisierung 3. Erwachsenen- und Hundehämoglobin nach Hybridisierung 4 Erwachsenen- und Hundehämoglobin nicht hybridisiert.

Bei der *Säuredenaturierung* wurde nach dem Prinzip der kinetischen Messung der Alkalidenaturierung verfahren. 2,6 ml der dialysierten und ungepufferten Hämolysate wurden mit 0,1 ml einer /10 HCl versetzt und die Extinktionsänderung durch Umwandlung in salzsaures Hämatin bei 571 nm mit der Stoppuhr erfolgt.

Die *Hitzedenaturierung* wurde in Anlehnung an die Methode von Be ... und Grasnacht (16) durchgeführt. J 2,0 ml der während der Dialyse in Cyanhämoglobin überführten und auf pH 6,8 gepufferten Eluate wurden in gleichstarke Reagenzgläser gegeben, bei 60,5 C min ... geschüttelt und bei dieser Temperatur entsprechende Zeiten im Höppler Thermostaten belassen. Nach Herausnahme erfolgte die Abkühlung der Proben in Eiswasser Der denaturierte Farbstoffanteil wurde bei 6000 U min 10 min lang abzentrifugiert und die Konzentration des nicht denaturierten Anteils in 2 cm Mikroküvetten im Elko III bei Filter S 53 im Vergleich zur Ausgangskonzentration bestimmt.

Die *Oxydation* der mit / Vol. Tris-Maleat Puffer 0,2 m auf pH 6,0 und ... Hämoglobin eingestellten Eluate mit Kaliumferricyanid und Natriumnitrit erfolgte nach den Methoden von Betke et al. (20, 21) Die K_3FeCN Konzentration betrug 3,90 10 Mol l bei 2,66 10 Äqu. l Hämoglobin, d. h. Kaliumferricyanid war in 13,5fachem Überschuß der zur Oxydation erforderlichen Menge im Ansatz vorhanden. Für das Natriumnitrit wählten wir einen ...fachen Überschuß.

Ergebnisse

1 Bei der elektrophoretischen Trennung der rekombinierten Präparationen von HbA und Hbcan stellen sich wie zu erwarten, vier Fraktionen dar Bei der Verwendung von Nabelschnurhämolysat erhalten wir insgesamt sechs Fraktionen da sich im Blut des

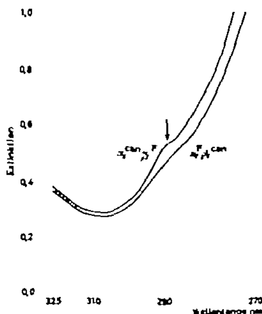


Abb. 2. UV-Spektrum der Hybride $\alpha_2^{can}\gamma_2^F$ und $\alpha_2^F\beta_2^{can}$. Der Pfeil zeigt auf die Tryptophanbande

Neugeborenen neben 50–70 % HbF noch entsprechende Mengen an HbA finden. Im allgemeinen kommen jedoch nur fünf Fraktionen zur Darstellung, da HbF und Hbcan praktisch keine Unterschiede in der elektrophoretischen Laufgeschwindigkeit aufweisen (Abb. 1). Betrachtet man die Verhältnisse bei den für die vergleichenden Untersuchungen in Frage kommenden Hybride, so sieht man, daß die $\alpha_2^{can}\gamma_2^F$-Kombination langsamer läuft als das entsprechende $\alpha_2^{can}\beta_2^A$-Hybrid.

2. Die Spektralanalysen im UV-Bereich lassen nur bei dem $\alpha_2^{can}\gamma_2^F$-Hybrid eine Tryptophanbande erkennen, die in der Gegend von 290 nm deutlich zur Ausprägung kommt. Die übrigen Kombinationen zeigen in diesem Bereich praktisch einen steilen geradlinigen Verlauf, wie er im allgemeinen für Hämoglobine charakteristisch ist (Abb. 2).

3. Die Kinetik der Alkalidenaturierung (Abb. 3) ergibt bei den eluierten Ausgangshämolysaten die bekannten Unterschiede zwischen HbA und HbF. Zwischen beiden liegt die Kurve des Hundehämoglobins. Sie ist identisch mit der Kurve der beiden (aus Erwachsenen-Hb bzw. aus Nabelschnur-Hb entstandenen) Hybride

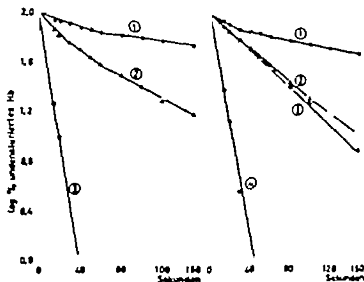


Abb 3. Kinetik der Alkalidenaturierung) ◯ Nabelschnurhämolysat, ◯ Hunde-hamolysat ◯ Erwachsenenhamolysat, b) ◯

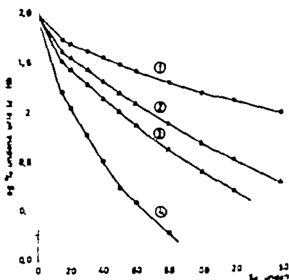


Abb 4 Kinetik der Säuredenaturierung

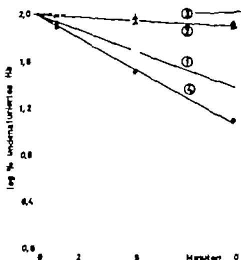


Abb. 5. Hitzedenaturierung: ① $\alpha^{can}\gamma^{F}$ ② $\alpha^{A}\beta^{can}$ ③ $\alpha_2^{F}\beta^{can}$ ④ $\alpha^{can}\beta^{A}$

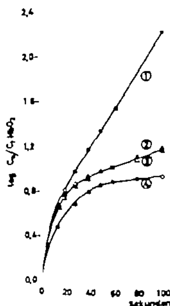


Abb. 6. Die Oxydationsgeschwindigkeiten der Hybride mit Kaliumferricyanid. Auf der Ordinate ist der Logarithmus C_0/C_t aufgetragen (Prozent OxyHb zu Beginn des Versuches dividiert durch Prozent OxyHb zu den jeweils angegebenen Zeiten)
① $\alpha^{can}\gamma^{F}$ ② β^{can} ③ $\alpha^{F}\beta^{can}$ ④ $\alpha^{can}\beta$

$\alpha_2\beta_2^{can}$. Dagegen weist die Kombination $\alpha_2^{can}\gamma_2^F$ eine ausgesprochene Alkalistabilität auf, während das zu vergleichende Hybrid $\alpha_2^{can}\beta_2^A$ sich wie HbA verhält.

4. Bei der Säuredenaturierung (Abb. 4) besitzt die γ_2^F-Kombination entsprechend dem fetalen Hämoglobin eine wesentlich größere Resistenz als das $\alpha_2^{can}\beta_2^A$-Hybrid, welches wiederum rascher denaturiert wird als die beiden α-Kombinationen von HbF und HbA mit Hundehämoglobin.

5. Die Ergebnisse der Hitzedenaturierung sind in Abb. 5 zusammengestellt. Die beiden Kombinationen der α-Ketten von Erwachsenen bzw. Nabelschnur-Hämoglobin mit β_2^{can} erweisen sich als die resistentesten Fraktionen. Von den beiden schnell wandernden Hybriden zeichnet sich $\alpha_2^{can}\gamma_2^F$ gegenüber $\alpha_2^{can}\beta_2^A$ durch die größere Stabilität aus.

6. Bei der Oxydation mit Kaliumferricyanid ergeben sich zwischen den einzelnen Hybriden erhebliche Differenzen (Abb. 6). Alle Kettenkombinationen zeigen in den ersten 20–30 sec mit graduellen Unterschieden im Prinzip eine initial rasche Oxydation, während die weitere Umwandlung der Hybride in Methämoglobin unterschiedlich langsam vor sich geht. Dadurch entstehen mehr oder weniger stark gekrümmte Kurvenverläufe. In den α-Kettenhybriden von HbA und HbF erfolgt die Oxydation in

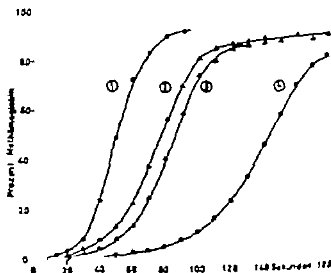


Abb. 7 Die Oxydationsgeschwindigkeiten der Hybride in Natriumnitrit [illegible]

quantitativ und qualitativ gleichem Ausmaß. Das α_2^{Can} / γ_2^F Hybrid wird sehr rasch, die α_2^{Can} β_2^A Kombination nur sehr langsam oxydiert. Wenn auch der geknickte Kurvenverlauf keine exakten Angaben hinsichtlich des 50 %-Oxydationswertes erlaubt, so ergibt sich aus der Betrachtung des zeitlichen Ablaufes, daß sich die Oxydationsgeschwindigkeiten 1 min nach Zugabe von Kaliumferricyanid für die β_2^A und γ_2^F Kombination wie 1 : 17 verhalten.

7. Die Oxydation mit Natriumnitrit (Abb. 7) zeigt den bekannten S-förmigen Kurvenverlauf (21, 27). Das γ^F Hybrid wird am schnellsten, das β_2^A Hybrid am langsamsten oxydiert. Dazwischen liegen in guter Übereinstimmung die beiden α-Kettenhybride der Hämolysate aus Erwachsenen- und Nabelschnurblut. Tab. I zeigt die relativen Oxydationsgeschwindigkeiten bei 50 %iger Oxydation. Vergleicht man das β_2^A und γ_2^F Hybrid miteinander, so verhalten sich die relativen Oxydationsgeschwindigkeiten wie 1 : 4,5.

Tabelle I

Hybrid	α^{AF}	β^A	γ^F
Sekunden 50% Oxydat.	120	224	50
relativ. Oxydat.-Geschw.	1	0,53	2,4

Vergleich der Oxydationsgeschwindigkeiten der Hybride mit Natriumnitrit.

Diskussion

Mit Hilfe der Hybridisierung gelingt es, asymmetrisch dissoziierte Ketten eines Hämoglobins mit entsprechenden Ketten eines anderen Hämoglobins zu kombinieren. Diese Methode findet vor allem zur Differenzierung anomaler Hämoglobine Verwendung, wobei aufgrund der von der Norm abweichenden Wanderungsgeschwindigkeit oder dem spektralen Verhalten (bei HbM Anomalien) der Hybride sicher entschieden werden kann, in welcher Kette die Anomalie liegt.

Von Huehns et al. (24) wurden erstmals vergleichend die Eigenschaften der Hybride im Hinblick auf die für das fetale Hämoglobin typische Alkalistabilität und die Tryptophanbande untersucht. Unsere Ergebnisse stellen eine Bestätigung dieser Befunde dar. Die Tryptophanbande, deren Ausprägung durch das Verhältnis von Tryptophan zu Tyrosin in der Polypeptidkette bestimmt wird, zeigt bei unseren Analysen für die γ_2^F Kombination eine deutliche Schulter in dem Bereich zwischen 290,5 und 288,8 nm, während Huehns et al. (24) einen Wert von 290,2 nm angeben.

Das reine fetale Hämoglobin hat ein Maximum bei 289,6 nm, bei HbA kommt es angedeutet bei 291 nm zum Vorschein (18). Die ausgesprochene Alkaliresistenz von HbF ist ebenfalls eine Funktion der Anwesenheit der γ-Kette, wie aus dem kinetischen Ablauf der Alkalidenaturierung der einzelnen Hybride hervorgeht. Die Tatsache, daß fetales Hämoglobin und das entsprechende Hybrid keinen Kurvenverlauf zeigen, der sich aus einem labilen und stabilen Anteil zusammensetzt, wird als Ausdruck einer symmetrischen Kettenspaltung ($2\ \alpha\ \gamma$) unter der Alkalieinwirkung gedeutet (24).

Von den weiter durchgeführten Denaturierungsmaßnahmen entsprechen die Ergebnisse bei der Säuredenaturierung den Erwartungen. Wie auch das fetale Hämoglobin, besitzt das γ_2^F Hybrid eine wesentlich größere Resistenz als das vergleichbare β_2^A Hybrid gegenüber Säureeinwirkung. Die relativen Geschwindigkeiten bei 50 % denaturiertem Farbstoff verhalten sich wie 4 : 1. Schwieriger ist das Ergebnis der Hitzedenaturierung zu interpretieren. Fetales Hämoglobin wird unter den gleichen Versuchsbedingungen gut doppelt so rasch denaturiert wie HbA des Erwachsenen. Vergleicht man das Ausmaß der Denaturierung der einzelnen Hybride, so erweist sich die β_2^A Kombination im Vergleich zum γ_2^F Hybrid als die labilere Fraktion. Da die α_2^{can} Ketten in beiden Präparationen die gleichen sind, kann für das Verhalten eine größere Empfindlichkeit der β-Kette vermutet werden. Diese Annahme findet Unterstützung in der ausgeprägten Hitzelabilität von HbH (β_4) (28) und dem Vergleich von HbA mit den β_2^{can} Hybriden, die identische α-Ketten von HbA und HbF besitzen. Das Fehlen eines zu erwartenden geknickten Kurvenverlaufes könnte durch symmetrische Zerstörung der Ketten gedeutet werden.

Die Oxydation von Hämoglobin mit K_3FeCN_6 in Hämolysaten läuft normalerweise wie eine monomolekulare Reaktion ab, deren Geschwindigkeit eine Funktion der Sauerstoffdissoziation ist. Bei der Auswertung der Ergebnisse der Oxydationen mit Kaliumferricyanid erhalten wir jedoch einen geknickten Kurvenverlauf, der in ähnlicher Weise bei verschiedenen Tieren (29) gefunden wird. Zur Erklärung der veränderten Form der Oxydationskurven bieten sich im wesentlichen 3 Möglichkeiten an. Einmal kann man vermuten, daß durch die Hybridisierung selbst Schaden am Hämoglobinmolekül entstanden sind, die die O_2 Dissoziation verändern. Eine weitere Möglichkeit liegt darin, daß die einzelnen Ketten der

Hybride mit unterschiedlicher Geschwindigkeit oxydiert werden, wie MARTIN UND HUEHNS (34) aus dem Kurvenverlauf bei der Nitritoxydation verschiedener Hämoglobine abgeleitet haben. Letztlich muß bedacht werden, daß die Hybridisierungsexperimente zum Schutz des Hämoglobins vor Zerstörung in CO-Atmosphäre durchgeführt wurden. Es besteht die Möglichkeit, daß trotz langzeitiger Elektrophorese und Dialyse noch ein Teil des Blutfarbstoffs als CO-Hämoglobin vorgelegen hat. Der Ablauf der Oxydation würde dann durch die langsamere CO-Dissoziation bestimmt. Für den grundsätzlichen Aussagewert dieser Befunde dürfte das jedoch ohne Bedeutung sein, da alle Präparationen und Oxydationsexperimente unter gleichen Bedingungen durchgeführt wurden.

Bei der Nitritoxydation erhalten wir den zu erwartenden S-förmigen Kurvenverlauf. Der raschen Oxydierbarkeit des γ_2^F Hybrids steht die langsame Oxydation der β_2 Kombination gegenüber. Dieses Ergebnis stellt einmal im Hinblick auf die unterschiedliche Oxydierbarkeit der Hybride im Prinzip eine Bestätigung der Befunde der K_3FeNC_6-Oxydation dar. Zum anderen entsprechen die Oxydationsgeschwindigkeiten, wenn man die beiden $\alpha_2^{A,F}$ β_2^{can} Hybride mit der γ_2^F Kombination vergleicht, quantitativ fast den Unterschieden zwischen HbF und HbA, deren Reaktionsgeschwindigkeiten sich wie 2 : 1 verhalten. Die auffallende Reaktivität des β^A Hybrids bei den Oxydationsexperimenten und der Hitzedenaturierung bedarf im Hinblick auf die für Funktion und Stabilität der in der β-Kette lokalisierten reaktiven SH-Gruppen (30–34) weiterer Untersuchungen.

Zusammenfassung

Zur Klärung der verschiedenen besonderen Eigenschaften des fetalen Hämoglobins wurden die Hybride von Erwachsenen- und Nabelschnurhämolysat in bezug auf folgende physikalisch-chemische und funktionelle Eigenschaften untersucht: Ultraviolett-Spektrum, Ablauf der Alkalidenaturierung, der Säuredenaturierung und der Hitzedenaturierung, Ablauf der Oxydation mit Natriumnitrit und mit Kaliumferricyanid. Die Ergebnisse lassen den Schluß zu, daß die Eigenschaften der Hybride durch entsprechenden Vergleich als Eigenschaften der Kettenpaare identifiziert werden können. Das γ-Kettenhybrid verhielt sich mit Ausnahme der Hitzedenaturierung bei allen experimentellen Prüfungen qualitativ wie fetales Hämoglobin. Aufgrund des gleichen Verhaltens der α-Kettenhybride von HbA und HbF wird die Identität der α-Ketten beider Hämoglobine auch funktionell bestätigt.

Summary

In order to elucidate the properties of foetal haemoglobin, the following physico-chemical and functional characteristics of hybrids of adult and cord blood haemolysate

Das reine fetale Hämoglobin hat ein Maximum bei 289,6 nm, bei HbA kommt es angedeutet bei 291 nm zum Vorschein (18). Die ausgesprochene Alkaliresistenz von HbF ist ebenfalls eine Funktion der Anwesenheit der γ-Kette, wie aus dem kinetischen Ablauf der Alkalidenaturierung der einzelnen Hybride hervorgeht. Die Tatsache, daß fetales Hämoglobin und das entsprechende Hybrid keinen Kurvenverlauf zeigen, der sich aus einem labilen und stabilen Anteil zusammensetzt, wird als Ausdruck einer symmetrischen Kettenspaltung (2 α γ) unter der Alkalieinwirkung gedeutet (24).

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Die Oxydation von Hämoglobin mit K_3FeCN_6 in Hämolysaten läuft normalerweise wie eine monomolekulare Reaktion ab, deren Geschwindigkeit eine Funktion der Sauerstoffdissoziation ist. Bei der Auswertung der Ergebnisse der Oxydationen mit Kaliumferricyanid erhalten wir jedoch einen geknickten Kurvenverlauf, der in ähnlicher Weise bei verschiedenen Tieren (29) gefunden wird. Zur Erklärung der veränderten Form der Oxydationskurven bieten sich im wesentlichen 3 Möglichkeiten an. Einmal kann man vermuten, daß durch die Hybridisierung selbst Schäden am Hämoglobinmolekül entstanden sind, die die O_2-Dissoziation verändern. Eine weitere Möglichkeit liegt darin, daß die einzelnen Ketten ver-

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Acta haemat. 32: 292–298 (1964)

The Effect of the Use of Different Tissue Extracts on One-Stage Prothrombin Times*

By L. Poller** Manchester

Standards of anticoagulant treatment vary considerably between different hospitals. The most important factor which is difficult to control is the laboratory technique used in assessing the dosage of the oral coumarin indanedione drugs. Not only are there several alternative methods available, e. g. Quick prothrombin time, P+P test and thrombotest, but different reagents may give very great variation in the results with the same technique. One of the main variables is the tissue thromboplastin extract employed.

Quick (1935) used rabbit brain as the source of thromboplastin. Other animal brain extracts, brain and lung mixtures, and human brain thromboplastin extracts have been substituted in the Quick test. The effect of the use of a variety of tissue thromboplastins on prothrombin activity of patients on anticoagulant treatment has been studied and a comparison made between the therapeutic ranges advocated using the different thromboplastins.

Methods

A group of 13 thromboplastin extracts was studied in this investigation. It consists of 3 human brain preparations, 2 acetone dried and one saline extract, 7 commercial extracts in widespread use of which 5 were rabbit brain preparations (Dade, Difco, Stayne, *Diagen dried* and *Diagen phenolised*), 1 pig brain (Geigy), 1 rabbit brain and lung mixture (Simplastin) and a group of 3 reagents which incorporated adsorbed plasma.

A. Owren's Thrombotest (4) with ox brain, calcium, adsorbed plasma and cephalin.

B. 2, 7, 10 Reagent (3) with ox brain, calcium, and adsorbed plasma.

C. Withington capillary reagent (5) consisting of human brain, calcium and adsorbed human plasma.

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* This work was performed whilst in receipt of a research grant from Manchester Regional Hospital Board.

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In order to give uniformity in the expression of results, standard saline dilution curves were prepared for each of the thromboplastins with sheep plasma. This was chosen because of its availability in bulk. As satisfactory curv was not obtained with some of the thromboplastin extracts with the sheep plasma the procedure was repeated with all of them using pooled normal human plasma.

Specimens were obtained from 40 patients undergoing short-term anticoagulant therapy. In 30 patients single specimen was obtained, in further 10 patients specimens were taken throughout their course of treatment, particularly during the first few days when the main depression of prothrombin activity is often due to simply factor VII depression. The one-stage prothrombin time technique is measure of the extrinsic (tissue) clotting system and fails to register changes in factor IX (Christmas factor). In the 10 patients followed throughout the course of treatment, thromboplastin generation tests were performed using the method of BIGGS AND DOUGLAS (2) with BELL AND ALTON (1) platelet substitute. Thromboplastin concentration was estimated after 6 min. incubation. This was done to show whether any of the thromboplastin reagents showed appreciable sensitivity to changes in the intrinsic (blood) thromboplastin system.

Specimens were tested with our saline preparation of human brain and those at arious representati levels of prothrombin activity were then tested with the different thromboplastin extracts. The study was planned to minimise the effect of contact activation and storage on the resulting prothrombin times. The specimens were collected with sterile dry syringes into plastic containers with 1/10th volume of 3.8% sodium citrate. Where batch of tests was performed each specimen was tested in turn with the complete range of thromboplastins before the next specimen was considered.

Observations were made on the therapeutic range stated by the manufacturers. This was compared with the range advocated with our human brain preparation, the latter being based upon the clinical experience over period of several years.

Additional features studied were sensitivity to factor VII depression. Insensitivity to factor VII ascribed to "factor VII contamination has been reported with arious animal brain thromboplastins. With the 3 reagents containing adsorbed plasma the effect of contact activation on prothrombin activity was studied. Specimens were taken into glass and plastic containers and tested after one hour and again after 18 hours at 4 °C.

The one-stage prothrombin technique was employed for all investigations except when using 2, 7 10 OWREN' Thrombotest and the Withington capillary reagent where the appropriate method was used. 3.8% trisodium citrate anticoagulant 1 vol. to 9 ols. of blood used with M/40 $CaCl_2$ except where M/50 stated by manufacturers. Specimens were all collected into plastic containers. The thromboplastins were substituted in turn, the time for completion of all tests on an individual plasma was less than ½ hour so the effects of storage may be ignored.

The thromboplastins were prepared according to the manufacturers' instructions and results expressed as percentage prothrombin activity from the graphs provided, these having been checked by us. The saline extract of brain was prepared using the method of OWREN (modified 6)

T investigate sensitivity to factor VII plasma was obtained from patients in the early stages of anticoagulant therapy with prolonged QUICK tests (less than 10% activity) restored by addition of stored serum and whose T G T was normal. This was, therefore, presumed to be pure VII deficiency. The effect of the addition of dilutions 1–20 and 1 10 of stored normal serum on the results of the prothrombin activity of the factor VII deficient plasma was measured using the different thromboplastins.

Results

Sheep plasma dilution curves The human brain extracts all gave the expected dilution curve with sheep plasma but the commercial reagents dilution curves were all unsatisfactory being flat and failing to indicate the appropriate dilution. This was assumed to be evidence of species specificity with the animal tissue extracts. The saline dilution curves with pooled normal human plasma coincided in all cases with the graphs provided by the manufacturers.

Table I

Mean results with different thromboplastins in 30 patients.

Adsorbed Plasma Reagents	Mean activity %	Recommended therapeutic range %	Mean [illegible]
W. llington	15.4	5–70	2.3
2.7 10	18.9	10–20	1.5
Thrombotest	14.3	10–25	[illegible]
Other Reagents			
Human brain (saline)	[illegible]	10–30	2.0
Human brain (acetone)	31.2	15–30	1.8
Human brain (?) (acetone)	30.2	15–30	1.7
Diagen Phenolised	30.8	15–25	[illegible]
Diagen Dried	40.4	15–25	1.6
Stayne	76.1	Not stated	[illegible]
Dade	24.6	Not stated	1.8
Difco	27.8	Not stated	1.8
Simplastin	30.5	Not stated	1.5
Geigy	31.5	10–30	1.3

The mean results of the tests appear to be of the same order in percentage activity (table I) in all but one of the 10 extracts without added adsorbed plasma. There was one important exception Diagen, dried reagent, gave much higher results. All 3 preparations containing adsorbed plasma gave much lower results in percentage activity The difference between the highest mean with Diagen, and lowest mean with thrombotest exceeded [illegible]. The use of the term prothrombin ratio which is

$$\frac{\text{prothrombin time of patients}}{\text{prothrombin time of the control}}$$

shows no appreciable advantage Ranges of 1.3 to 2.3 were obtained when ratios were substituted for activity with the various thromboplastin reagents.

Table II

Effect of storage in glass and plastic containers with adsorbed plasma reagents.

		Withington		2.7 10		Owren	Thrombotest
		Glass	Plastic	Glass	Plastic	Glass	Plastic
Specimen 1	1 hour	41	43	52	54	70	76 secs.
	18 hours	39	48	39	56	48	76
Specimen 2	1 hour	63	66	72	76	88	91
	18 hours	57	67	52.5	80	59	97

Therapeutic ranges of 15—25 / and 18—30 / were provided with two commercial preparations without further data (table I)

All reagents showed reasonable sensitivity to factor VII levels and all showed depression during the first 48 hours of therapy

Specimens stored in plastic containers showed no significant shortening over an 18 hour period. Thrombotest and 2.7 10 gave considerably shortened times by storage in glass whereas the Withington reagent showed little change. Thrombotest showed contact activation even when stored in plastic containers. No appreciable sensitivity to alteration in the intrinsic thromboplastin system was found with any reagent.

Discussion

Effectiveness of anticoagulant treatment depends not only on the skill of dosage of anticoagulant drugs but also on the degree of depression of coagulability The latter is to a large extent dependent on the laboratory technique and reagents employed in its control. The results of prothrombin estimations may be expressed in many ways, e.g as a ratio

$$\frac{\text{prothrombin time of patients}}{\text{normal}}$$

prothrombin activity (from many different types of dilution curve) or prothrombin index. All of these procedures introduce variables which are difficult to standardise and produce a lack of uniformity of treatment between hospitals. We have used the term "prothrombin activity" throughout so that valid comparisons could be made between the reagents. That little is gained by the alternative term ratio may be seen in the table. Extremes of 1.3 to 2.3 are seen. The fact that the relationship between prothrombin time and activity is not linear is ignored when this system is used.

One of the main sources of variation is the thromboplastin reagent employed in the prothrombin estimation. Many types of

thromboplastin extract are available commercially none of which are identical and where hospitals prepare their own extracts these often differ quite considerably

In the present study we have included a group of selected commercial thromboplastins which are widely used and have been thoroughly tested in clinical practice, against a group of individually prepared hospital thromboplastin reagents. Although all of these reagents appear to be reasonably satisfactory for clinical use important differences in the percentage of prothrombin activity and ratio were found.

The optimum range of depression of the Quick test to ensure control of thrombo-embolic disease may be determined by two methods. Originally the depression of prothrombin activity required to prevent artificially induced thrombosis in animals was observed. A second and more convincing approach has been the determination of the coagulation defect needed to secure a good result in man in the treatment and prophylaxis of thromboembolism. Many clinicians have arbitrarily chosen a range of activity of 15—30 / regardless of the type of thromboplastin used and the type of dilution curve, others have taken a ratio of 1½—3 times the normal value or an index of 40—60 / as the therapeutic range. This can only be gauged by the proof of the clinical value using a particular batch of thromboplastin of a given therapeutic range of activity ratio, or index, or by parallel observations with an established reagent.

Provided due note is taken of the fact that all thromboplastins must be assessed individually the use of reagents such as Diagen which gives much higher percentage activity results and the 3 adsorbed plasma reagents which give much lower results might be justified. The therapeutic range with the latter group must of course be considerably lower than 15—30 / (with the optimum probably in the region of 8—15 %) This is the range advised with the Withington reagent (5) and not greatly different from the range of the 2.7 10 reagent, (10—20 %) because this gave slightly higher results but is somewaht less than the 10—25 / advocated for thrombotest. The lower results in percentage activity with these 3 reagents arises from the incorporation in them of an excess of adsorbed plasma. This gives dilution curves similar to the adsorbed plasma dilution curves which are flatter than saline curves. The lower results would, therefore, be expected on first principles. In

contrast, with the Diagen D reagent the optimum range must be considerably higher than the 15—25 / advocated for this product.

The use of human brain does not have any obvious advantage over the best commercial extracts. As expected none of the one-stage reagents showed appreciable sensitivity to changes in the intrinsic clotting system. The precise relevance of reduction of factor IX by coumarin and indanedione drugs remains to be established, both in relation to treatment and haemorrhage.

The question of factor VII sensitivity is of importance because this may be depressed first by oral anticoagulants. All of the reagents tested could safely be used for controlling anticoagulant therapy since they all showed reasonable sensitivity to factor VII levels. Verstraete et al. (7) found rabbit brains were unsatisfactory due to 'factor VII contamination'. We have not found any evidence of this although we found lack of sensitivity due to species specificity with some commercial thromboplastins when tested with sheep plasma.

If clinical trials or results of treatment are to be satisfactory when conducted at different centres standardisation of reliable thromboplastin extracts is of upmost priority. A large supply of thromboplastin can be prepared and then be distributed to a group of hospitals or a large region. Such a scheme is already in operation covering a population area of several millions in the Manchester Region. Such a scheme should not only facilitate research but allows hospital specialists and general practitioners to share a common basis for their dosage of anticoagulant drugs in different laboratories by which they are served.

Acknowledgements. I wish to thank Sylvia M. Radfern for technical assistance with this work.

Summary

The effect of the use of a variety of thromboplastin reagents on the results of the one-stage prothrombin determinations has been studied. These consisted of 3 human brains, 5 animal extracts and 3 reagents which incorporate adsorbed plasma—Wikbington, 2, 7, 10 and thrombotest. Nine of 10 tissue extracts gave results of the same order. The three more complex adsorbed plasma reagents were alike in their results but their therapeutic range appeared to be substantially lower in percentage activity.

The therapeutic range with every type of thromboplastin must be established individually, either on the basis of clinical experience or parallel observation with a standard reagent. Standardisation of thromboplastin reagent is necessary, not only for clinical trials but also that there may be a common basis for dosage of anticoagulant drugs.

Experimental

Packed cells have been prepared from anticoagulated blood obtained from human, rats and sheep. Blood was centrifugated at 4 °C for a period of 10 minutes at 13,500 r.p.m. (about 15,000 g). The supernatant was removed and the sample was centrifugated again at identical conditions. Few drops of supernatant were again removed. The haematocrit of such samples was checked subsequently by means of a high-speed microhaematocrit machine. Only traces of meniscus have been observed. On occasion the packed cells were diluted with saline and the procedure repeated.

The viscosity of packed cells has been determined by means of rotational concentric viscometer described formerly (31, 32).

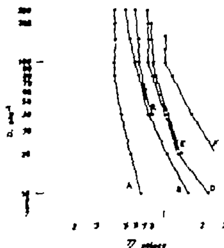


Fig. 1. Viscosity of packed red cells determined by means of rotational viscometer. Curves A, B, C, D, E and F correspond to packed cells of three-years old boy, sheep, an infant, rat, a polycythaemic patient, and sheep, respectively. Sample C was studied in saline, all others were centrifugated from plasma. Viscosity curves are plotted on a log-log scale, as viscosity η in poises, against the rate of shear D, in reciprocal seconds.

Suspensions of rigid particles were prepared by dispersing commercial pigments titanium dioxide and lead oxide in toluene in presence of small quantity of surface active agents. Suspensions were centrifugated and the concentrations adjusted. Viscosity of these suspensions has been determined by means of Brookfield Synchroelectric Viscometers, HBT models.

Viscometric techniques have been described formerly (28, 32).

Results and Discussion

Confirmation of the internal fluidity of the red cell. A series of packed red cells, of haematocrit values above 98 %, have been studied by means of rotational viscometer. The viscosity curves have been

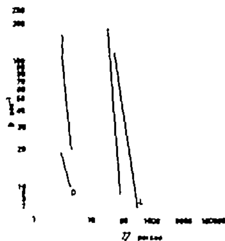


Fig. 2. Comparison of viscosity of suspensions of rigid particles (K, L, M, N) with viscosity of packed red cells (A, D, F). Packed cells correspond to the systems described in fig. 1. Suspensions of rigid particles are represented by titanium dioxide suspension in toluene at 66% packed volume (K, L), titanium dioxide suspension in toluene at 87% packed volume (M), and lead oxide suspension in toluene 85 packed volume.

Please observe that although the concentration of suspended rigid particles is much less than that in packed red cells (haematocrit 98) the viscosity is larger by factors from 200 to 500,000.

plotted over the range of rates of shear 9 to 250 sec^{-1} In all cases the viscosity slightly decreased when the velocity gradient increased (fig 1)

In order to observe the effect of rigidity of suspended particles on the viscosity of suspensions, four suspensions of titanium dioxide and lead oxide were studied and plotted (fig 2) Although these suspensions contained a much lower concentration (66 85 and 87 /) of the suspended particles than was the case with packed cells, the viscosities observed were 100-fold to 500 000-fold higher than the viscosity of blood at haematocrit of 98 /

Discrepancies in viscosity between blood and suspensions of any rigid particles (glass, oxides, clays, etc.) over the whole range of concentrations, are well documented as can be seen from the fig 3 Blood viscosity as a function of haematocrit value is plotted after HAYNES (44) in curve 3 after HATSCHECK (43) in curve 4 and after EARLEY AND ATWATER (35) in curve 5 Similar viscosity values with changing haematocrit have been reported by WELLS AND

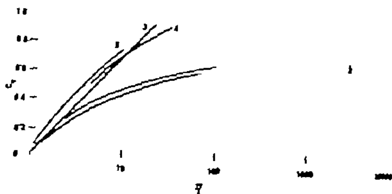


Fig 3. Relative viscosity of blood (curves 3, 4 and 5) and of suspensions of rigid particles (curves 1 and 2). Viscosity of blood as a function of the haematocrit value (expressed here as volume fractions C_v) is plotted from published data of HAYNES (44), of HARSCHEK (45) and of EARLEY AND ATWATER (35), as curves 3, 4 and 5, respectively. Curves 1 and 2 are plotted after DINTENFASS (25, 28) and ROSCOE (77) respectively. The viscosity data correspond to disaggregated suspensions and are thus not a result of non-Newtonian characteristics of suspensions.

MERRILL (97) and STRUMIA AND PHILLIPS (93). Curves 1 and 2 (fig 3) represent changes of viscosity in disaggregated suspensions of rigid particles as a function of concentration (volume concentration or volume at close packing) according to DINTENFASS (25, 28) and ROSCOE (77) respectively. At the concentration of about 65—75 %, depending on the shape of the particle, suspensions of rigid particles achieve consistency of concrete. If even one could describe such material in terms of simple viscosity it would be many billion-fold larger than the viscosity of blood at the same concentration of suspended cells.

There is only *one* possible explanation of the rheological behaviour of blood, and this is that blood should be treated as an emulsion or a suspension of fluid drops.

The fact that the viscosity of packed cells is as low as 0.4 to 2 poises, depending on the origin of the red cells, indicates that the interior of the red cell must be very fluid. As it is known from the theoretical works of TAYLOR (95) and OLDROYD (64, 65) that emulsions, in which the internal viscosity of the drop is about 50-fold of that of the continuous (suspending) fluid, behave already as suspensions of rigid particles, it must be obvious that the internal viscosity of the red cell must be greatly less than 30 centipoises. It has been already suggested (29) that the internal viscosity of the red cell can be expected to be between 2 and 20 centipoises.

This deduction, based solely on the rheological grounds, can be easily augmented by microscopic observations. Red cell in flow is known to undergoe rapid deformations (8) Branemark and Lindström (9) observed that a red cell caught in a narrow capillary segment performs a periodic protruding movement, like that of a fluid-filled bag Prothero and Burton (75 76) observed that red cells can pass steadily through microfilters of pores of only 3 microns. Small leucocytes were observed by Palmer (66) to indent red cells on contact, indicating that the leucocytes are more rigid than the red cells. Palmer observed also that red cells in flow assume often a tear-drop shape, with the tail of the drop trailing Bloch (7 8) using a high speed movie camera, noted that the deformation of red cells in flow occurs at a frequency in the order of milliseconds, which suggests again an extremely low internal viscosity

A question may arise what is the microstructure of the red cell interior. As the molecular weight of haemoglobin is about 64,000, and as its concentration is about 32%, the manner of packing and the physical states of haemoglobin would be very important. Again, on purely rheological grounds, it cannot be expected that haemoglobin is in true solution. Polymeric solutions of analogous molecular weights and concentrations would be expected to exhibit relative viscosity of about 100,000 (27 29). On the other hand, micro-crystalline or liquid-crystalline suspension of the same polymer at the same concentration might show relative viscosity of only 2 or 3. This deduction agrees well with the statement by Perutz (68) that arrangement of haemoglobin is intermediate between the order in a solid crystal and complete disorder in dilute solution. Presence of nematic phase in haemoglobin gels has been reported by Deaver et al. (20) while detailed discussions on crystalline and semi-crystalline phases of haemoglobin have been given by Porter (71, 73) and Francesco (74). A deduction that haemoglobin inside the red cell is not in true solution but in second phase (amorphous, paracrystalline, liquid-crystalline) is also augmented by an observation reported by Hammer (40) that haemoglobin exerts no osmotic effect.

The curves of viscosity of packed cells against the velocity gradient (fig 1) show a slight degree of thixotropy or shear thinning As this phenomenon is neither due to the minute quantity of free fluid (plasma or saline) present, nor it is due to the aggregation of the red cells, it must be ascribed to the structure of the red cell itself. Perhaps it would be more exact to state that the thixotropic effect is due to an integrated effect of the red cell interior and the red cell membrane.

Rheological effects of the red cell membrane. It can be fairly well accepted that the red cell behaves as a fluid drop. If the red cell were rigid (just as a rubber or plastic particle) the viscosity of blood, at haematocrits of 80 to 100% would be thousands and millions of times larger than it actually is. While the fluidity of the

interior of the cell is a necessary condition for the apparent fluidity of blood it is not the unique condition. The role of the membrane surrounding the cell interior has to be elucidated.

Flow of a drop depends on the presence or the absence of an interfacial film or a membrane. In the absence of an interfacial film, the stresses existing in a flowing system can be resolved (80) into the tangential and the normal stresses (where 'normal is a mathematical term) acting at the drop interface. The tangential stresses are assumed to be transmitted undiminished across the interface and thus establish a velocity gradient inside the drop. The normal stresses are responsible for deformation of the drop (47) Internal circulation (95) in the fluid drop is due to the transmission of both the tangential and the normal stresses. Fluid circulation inside the drop reduces the distortion of the flow pattern outside the drop and, hence, reduces the viscosity of the suspension.

In the case when an interfacial film surrounds the drop, the rheological characteristics of this film will influence the transfer of tangential and normal stresses. Films of high interfacial viscosity suppress the internal circulation in a drop, and cause such drop to behave as a rigid body (5 63 79 80 84 85) A visco-elastic film has similar effect (64 65)

It is known that simple fatty acids or surfactants, which are used to stabilise emulsions, form monolayers or multilayers of appreciable viscosity The viscosity of this interfacial layer affects, in its turn, the viscosity of emulsion. For instance (1), 35% emulsion of water in benzene shows relative viscosity of 1.84, 1.94 or 2.86, depending on the fact if Ca-oleate, glycerol ricinoleate, or mixture of Mg-sulfonate and Ca-dodecylsalicylate have been used as stabilizers. An emulsion of drops covered by completely rigid membrane, or suspension of rigid particles, at the same concentration, will show relative viscosity of about 3.4 to 3.8. This effect is much more pronounced at high concentrations. SHERMAN's data (84 85) indicate that an increase in the rigidity of an interfacial film might increase hundreds of times the viscosity of emulsions containing droplets comparable in size to the red cells. Experimental evidence on these phenomena has been accumulated by MASON et al. (5, 63, 80) Monolayers of lipids and/or proteins may be also quite rigid (15 49) and will, therefore, cause the drops to behave as more or less rigid bodies.

As the red cell membrane does not inhibit a transfer of the tangential stresses, nor it inhibits the internal circulation, it must have a very low surface viscosity In a complex multilayer film such low viscosity can be achieved most conveniently by a thixotropic surface system. Such system would liquify under stresses imposed. A small elastic component would be, however necessary to retain the integrity of the membrane.

Deduction of the micro-rheological structure of the membrane If the red cell is to exhibit fluidity its membrane must be also fluid. Unfortunately the direct rheological studies of the red cell membrane are not easy SEIFRIZ (82) stated that properties of the membrane change after it has been torn off the cell, the disrupted membrane becoming more rigid. MUDD AND MUDD (60) observed that the membrane is a plastic solid or a viscous liquid, while PONDER (72, 73) indicated that the membrane is non-uniform both in structure and in rigidity

The molecular organization of the membrane was reviewed and discussed by many (18, 45, 62, 71 86) but it has been based, mainly on the studies of ghosts. The direct electron-microscopic studies are not conclusive. Recently MUIR (61) using electron micrography observed clear rings containing dark centres which were, in places, packed in hexagonal pattern. Similar observations were made by DOURMASHKIN et al. (34). The hexagonal structure was due, however to the action of saponin and, thus, considered by BANGHAM AND HORNE (4), GLAUERT et al. (37), and HOBSON AND LUZZATI (48) as artifact.

There are, however indeed some indications that the membrane consists of some smaller structural units. Even DANIELLI (16, 17) agreed that some degree of discontinuity must exists in the laminar "sandwich" of lipids and proteins, and introduced "pores formed by protein lamellae. KUZIN AND TRICHER (57) suggested that the membrane possesses a mosaic structure and consists of loci formed by different substances.

It is easy enough to find evidence for the heterogeneity of cell membrane in cells other than the red cell. According to WEISS (96) the cell surface is mosaic of patches of supramolecular order and of different chemical and physical properties. ROTHSTEIN (78) suggested that the surfaces of cells are composed of complex molecular mosaics undergoing local variations. MITCHISON AND SWANN (59) observed variable viscous component in the membranes of some cells.

Surface heterogeneity and structural complexity of the red cell membrane would be more in line with the present knowledge of immunology (38, 91) or the well known selective permeability of the membrane (51 81 88)

A microrheological structure of the membrane could be envisaged, at this stage, as complex and heterogeneous. The details might be elucidated from the following considerations. According to JOLY (50) each rheological type of the surface film has a specific counterpart in the rheology of bulk fluid systems. JOLY observed non-Newtonian and thixotropic behaviour in surface films composed of lipids. Plasticity (thixotropy) of surface films of sodium soaps was observed by BURCIK (11) and FILATOVA et al. (36) It is rather

portions of the membrane becoming more viscous while other becoming simultaneously more fluid or more elastic.

A presence of microstructure in the membrane would favour heterogeneity of the cell surface. Indeed, heterogeneity of the red cell surface has been demonstrated in respect of antigen and antibody reactions (38) Heterogeneity of the membrane solves an apparent contradiction between observations that the red cell surface is polar or hydrophobic (60) A surface may be simultaneously hydrophobic and polar due to an existence of series of specific active sites. This type of the selective adsorption pattern has been demonstrated already on a multitude of non-biological surfaces (22, 23 24)

The modern permeability theories (51 81 88) fit quite well into the new model of the red cell membrane. Liquid-crystalline and micellar components could be expected to participate in transfer processes and in the processes of biochemical synthesis. It is worth noting that SOLLNER (88) stated that membranes act as physico-chemical machines they regulate the flow of the energetic processes which occur within their thickness, and in doing so, they transform various forms of energy into others. It is not yet known how these processes are affected by the mechano-chemical transformation principle (6, 12, 55 56, 92)

Conclusions

Rheological studies of suspensions of red cells and of whol blood indicate that the interior of the red cell is fluid. In or however for the red cell to exhibit fluid properties, the int circulation in the cell, and the transfer of tangential stresses, not be inhibited by a rigid membrane.

This finding leads to a deduction that the quadruple l layer of lipids and proteins, assumed up till now to represent membrane structure, might be too rigid. The membrane s exhibit considerable fluidity and in order to achieve th following tentative model is suggested. The internal two l the traditional model are replaced by a mesomorph crystalline) layer capable of exhibiting thixotropic p liquid-crystalline layer may be expected to be analo gonal structures discovered recently in lipids and ph STOEKENIUS (90) and LUZZATI AND HUSSON (58)

layers of the traditional model are retained, although they are suggested to be of very low surface concentration of protein. The loose protein structure will be analogous to dilatant dynamic net works and should supply elastic component to the membrane.

Such inherently heterogeneous and multiphase model of the red cell membrane might be expected to agree better with the present state of knowledge on immunology membrane permeability and enzymatic processes within the membrane.

Acknowledgment. I am indebted for support to the National Heart Foundation of Australia.

Summary

The study of viscosity of blood at high haematocrits and of packed cell systems leads to conclusion that the interior of the red cell is fluid. The internal viscosity is suggested to be in the range from 2 to 20 centipoises. If the red cell is to exhibit fluidity transfer of the tangential stresses (in flow) from the exterior to the interior of the red cell must not be inhibited by rigid membrane. Consequently the red cell membrane should be characterized by extremely low surface (interfacial) viscosity. It is suggested that feasible model of the red cell membrane could be formed by liquid-crystalline or micellar lattice arrangement of lipids and proteins. Such model would account naturally for the surface heterogeneity of the red cell and would supply basis for the catalytic chemical reactions and the active transport considered to exist in the membrane.

Résumé

L'étude de la viscosité de sang ayant un hématocrit elevé et des éléments cellulaires très serrés permet de conclure que l'intérieur des érythrocytes est fluide. La viscosité intérieur est de l'ordre de 2 à 20 centipoises. Si l'érythrocyte doit se comporter comme une masse liquide la transmission de forces tangentes (dans le courant) de l'exterieur à l'intérieur de l'érythrocyte ne doit pas être empêchée par une membrane rigide. Par conséquent la membrane des erythrocytes doit être caracterisée par une viscosité de surface extremement faible. Un modèle possible de la membrane du globule rouge serait fait d'un arrangement en forme de grille de lipides et de protéines d'un état liquide à cristallin ou micellaire. Un tel modèle tiendrait compte sans difficulté de la surface hetérogène de l'erythrocyte et fournirait une base aux réactions chimiques catalytiques et aux transports actifs qui doivent se faire dans la membrane.

Zusammenfassung

Die Untersuchung der Viskosität von Blut mit hohem Hämatokrit und gepackten Zellsystemen ergab, daß der Inhalt der Erythrozyten flüssig ist. Die innere Viskosität liegt im Bereich von 2 bis 20 Centipoise. Wenn das rote Blutkörperchen Flüssigkeit enthält, kann die Übertragung tangentialer Kräfte von aussen nach innen nicht durch eine starre Membran gehemmt sein. Daher muß die Erythrozytenmembran eine extrem niedrige Oberflächenviskosität besitzen. Es wird erwogen, daß ein Modell der Erythrozytenmembran aus einem flüssig-kristallinen oder micellaren Gitter von Lipoiden und Proteinen bestehen könnte. Ein solches Modell würde der heterogenen Oberfläche des Erythrozyten Rechnung tragen und eine Grundlage bilden für die katalytischen chemischen Reaktionen und den aktiven Transport in der Membran.

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Acta haemat. 32 314–320 (1964)

Aus der Medizinischen Universitätsklinik Innsbruck
(Vorstand Prof. Dr. H. Braunsteiner)

Angeborene hämolytische Anämie mit Mesobilifuscinurie und Innenkörperbildung nach Splenectomie

Von H. Braunsteiner, F. Dienstl, S. Sailer
und F. Sandhofer

In den letzten Jahren sind einige Fälle von angeborener hämolytischer Anämie beschrieben worden, die durch das Vorkommen eines sehr hohen Prozentsatzes von Erythrozyten mit Innenkörpern nach Splenektomie und durch das Auftreten eines dunkelbraunen bis schwärzlichen Harnes gekennzeichnet sind (6 9 11 15 7). Die Innenkörper sind morphologisch und in ihren Färbeeigenschaften den Heinzschen Innenkörpern ähnlich. Sie lassen sich in bis zu 90% der Erythrozyten nachweisen. Der dunkle Urin ist durch die Ausscheidung von Mesobilifuscin (12) bedingt (6 9). Die Beziehung dieses Dipyrrols zum Hämoglobinstoffwechsel ist nicht klar, es ist jedoch wahrscheinlich, daß bei den Patienten ein pathologischer Hämoglobinstoffwechsel in den Erythrozyten vorliegt. Nach Transfusion dieser Zellen an Normalpersonen wurde in deren Harn Mesobilifuscin nachgewiesen (9).

Bisher wurden insgesamt 6 Fälle mitgeteilt. Die Störung ist möglicherweise genetisch bedingt, da das Syndrom familiär bei Vater und Sohn beobachtet wurde (9). Der Mechanismus der Erkrankung ist unbekannt. Im Glukosestoffwechsel der Erythrozyten konnte keine Anomalie nachgewiesen werden. Auch in der Hämoglobinstruktur fand sich bisher keine sichere Abweichung. Therapeutisch wurde durch Splenektomie ein mäßig guter Erfolg erzielt. Die Hämolysezeichen gingen zurück, blieben jedoch in gewissem Ausmaß erhalten.

Wir beschreiben einen weiteren Fall dieser Erkrankung, welcher der erste im deutschsprachigen Schrifttum ist. Neben dem klinischen Interesse ist die Beobachtung auch von genetischer Bedeutung, da bei der nicht splenektomierten Tochter des Patienten ein

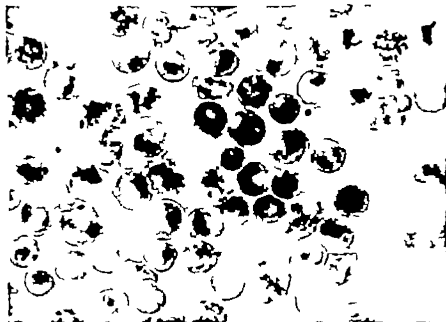


Abb. 1. Blutausstrich, Vitalfärbung mit Brillantkresylblau. Die Innenkörper in den Erythrozyten, die Retikulozyten und die normalen reifen Erythrozyten sind deutlich zu erkennen.

hämolytischer Prozeß mit nur vereinzeltem Auftreten von Innenkörpern beobachtet wurde.

Krankengeschichte

Es handelt sich um einen 31jährigen Patienten. Die Familienanamnese ist unauffällig: der Vater beging mit 49 Jahren wegen einer chronischen Osteomyelitis Suizid. Die Mutter, zwei Schwestern und ein Bruder sind gesund und weisen keinerlei Hämolysezeichen auf. Seit dem 3. Lebensjahr fielen den Angehörigen des Patienten Gelbfärbung der Haut und Skleren, dunkler Harn und gelegentlich subfebrile Temperaturen auf. Er wurde deshalb mehrfach in einem auswärtigen Krankenhaus aufgenommen und die Diagnose einer angeborenen hämolytischen Anämie gestellt. Als sich im Alter von 16 Jahren sein Zustand verschlimmerte und wiederholt Bluttransfusionen notwendig waren, wurde die Splenektomie durchgeführt. Seither hat sich sein Zustand klinisch gebessert. Es besteht ein mäßiggradiger Ikterus, schwere hämolytische Krisen traten aber nicht mehr auf.

Anläßlich einer Blutbildkontrolle an unserer Klinik fiel das massenhafte Auftreten von Innenkörpern auf. Bei der klinischen Durchuntersuchung zeigte der Patient bis auf die Gelbfärbung und die Narbe nach Splenektomie physikalisch keine Auffälligkeiten. Es bestand kein Turmschädel, keine Ulcera und keine Lebervergrößerung. Blutbild: Erythrozyten 3 450 000, Hämoglobin (Cyanhämiglobin-Methode) 9,5 g%, Hb$_E$ 27 γγ, Leukozyten 11 000, Differentialblutbild unauffällig, Thrombozyten normal, Retikulozyten 1,0‰. In 300 bis 400‰ der roten Blutzellen fand sich ein, selten mehrere unregelmäßig konturierte, etwa 1 bis 2 µ große Innenkörper (Abb. 1), die sich mit Nilblausulfat, Brillantkresylblau und Methylviolett anfärbten. Sie waren auch

phasenoptisch gut nachweisbar. Weiterhin fanden sich einzelne Erythrozyten mit basophiler Tüpfelung und solche mit Howell-Jolly-Körperchen. Der mittlere Erythrozytendurchmesser betrug 6,8 µ, das Serumeisen 160 γ%, das freie Eisenbindungsvermögen 150 γ%. Blockungtest und Coombs-Test waren negativ, freies Haptoglobin war nicht nachweisbar. Im Sternalmark fanden sich die Zeichen einer ausgeprägten erythropoetischen Hyperplasie. In den oxyphilen Normoblasten ließen sich vereinzelt Innenkörper nachweisen. Weiterhin fanden sich zahlreiche Sideroblasten und pigmentierte Makrophagen. Autohämolyse (16) 2,3 (normal). Osmotische Resistenz (8): 50%ige Hämolyse 0,415%. Auffällig ist der hohe Anteil an besonders resistenten Zellen, wie schon von Schmid et al. (9) beobachtet (Abb. 2).

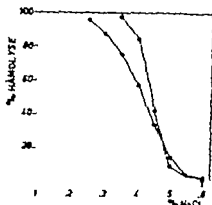


Abb. 2. Osmotische Resistenz der Erythrozyten. ●———● Erythrozyten des Patienten. o———o Normale Erythrozyten. Der Anteil der osmotisch besonders resistenten Zellen ist erhöht.

Enzymbestimmungen wurden im optischen Test im Photometer Eppendorf mit elektronischem Registrierzusatz bei 25 °C bestimmt. Die Aktivitäten werden in internationalen Einheiten (µMole Substratumsatz/Minute) bezogen auf 10^{10} Erythrozyten (nach Isolierung mittels Dextran) angegeben. Die Glucose-6-Phosphatdehydrogenase-Aktivität (4) betrug 384 I.U. (deutlich erhöht), die Pyruvatkinaseaktivität (14) war 341 I.U. (normal), die 6-Phosphogluconatdehydrogenase-Aktivität (4) war mit 314 I.U. mäßig erhöht, die 2,3-Phosphoglyceromutase-Aktivität (13, Modifikation nach Kochen et al., 5) betrug 120 I.U. (normal), die TPNH-abhängige Glutathion(GSSG)-Reduktase (10) 106 I.U. (normal) und die DPNH-abhängige Glutathion(GSSG)-Reduktase (10) 45 I.U. (normal). Das Glutathion (1) betrug 46 mg GSH/100 ml Erythrozyten (untere Grenze der Norm), das Methämoglobin (2) 0%. Die Stärkegel-Elektrophorese des Hämoglobins ergab keinen sicheren pathologischen Befund (Boratpuffer pH 8,7).

Sämtliche Leberfunktionsproben lagen in der Norm, ebenso die [illegible]proben. Das Serumbilirubin schwankte zwischen 1,8 und 2,5 mg%. Die Papierelektrophorese des Serums war unauffällig. Thorax-Röntgen und EKG o.B.

Die Untersuchung des Harnes des Patienten, die wir Herrn Prof. Dr. W. Siedel, Frankfurt (M)-Höchst, verdanken, und dem wir an dieser Stelle herzlich danken wollen, ergab folgendes Resultat: Der Vergleich des Harnes des Patienten mit normalen Harnen zeigt, daß er etwa das 3,5fache der Bilirubinabbauprodukte enthält, das zum Teil spontan in Bilifuscin bzw. Mesobilifuscin übergeht, bzw. mit HCl in diese Verbindungen überführbar ist, wenn man die Menge an Bilifuscin- oder Mesobilifuscinvorstufen in normalem Harn gleich 1 setzt. Der untersuchte Harn hat von allen bisher von uns untersuchten Harnen den höchsten Gehalt an Bilifuscinen und deren Vorstufen.

Die Untersuchung des Hämoglobinstoffwechsels durch Bestimmung der Erythrozytenporphyrine und ihrer Vorstufen, die wir Herrn Doz. Dr. Clotten, Med. Univ. Klinik Freiburg i. Breisgau, Deutschland, verdanken, ergab folgende Werte: Koproporphyrin 11,5 γ/100 ml, Protoporphyrin 65 γ/100 ml, Porphobilinogen 40 γ/100 ml, δ-Aminolävulinsäure 55 γ/100 ml. Alle Werte waren somit deutlich erhöht, ansonsten aber ähneln sie den auch bei anderen hämolytischen Anämien zu erhebenden Befunden.

Bei der 6jährigen Tochter des Patienten fand sich folgendes Blutbild: Erythrozyten 4 000 000, Hämoglobin 10,6 g%, Hbe 27 μμg, Leukozyten 11 600 mit normalem Differentialblutbild. Die Retikulozyten betrugen 250 ‰. In 15‰ der Erythrozyten wurden Innenkörper nachgewiesen. Serumbilirubin 1,4 mg%, Coombs-Test negativ, kein freies Haptoglobin nachweisbar.

Diskussion

Der von uns mitgeteilte Fall entspricht in der Klinik und im Ergebnis der Laboratoriumsuntersuchungen völlig den von Lange and Akeroyd (6), Schmid et al. (9), Worms et al. (15), Scott et al. (11) und Mozziconacci et al. (7) beschriebenen Fällen. Dieses Krankheitsbild kann heute somit als gut umschriebenes Syndrom gelten. Die Ätiologie dieser Erkrankung ist noch ungeklärt. Ob die von Scott et al. (11) beobachtete elektrophoretisch nachgewiesene Hämoglobinabnormalität tatsächlich eine spezifische pathogenetische Bedeutung besitzt, muß erst durch entsprechende genaue Untersuchung der übrigen geschilderten Fälle geklärt werden.*

Die bis jetzt durchgeführten Untersuchungen der Enzyme des Kohlehydratstoffwechsels, von denen bekannt ist, daß durch ent

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Anmerkung bei der Korrektur: Seit der Einsendung des Manuskriptes wurde von drei verschiedenen Arbeitsgruppen bei einem gleichartigen Krankheitsbild ein abnormes Hämoglobin nachgewiesen. S. Shibata, I. Iuchi, T. Miyaji, S. Ueda und I. Takeda: Hemolytic disease associated with the production of abnormal hemoglobin and intraerythrocytic Heinz bodies. Acta haemat. jap. 26: 164–173 (1963). Durch Elektrophorese (Agargel, Papier, Stärkeblock), Chromatographie (Amberlite IRC 50 und Carboxymethylcellulose), Fingerprint-Technik und Titration der SH-Gruppen mit p-Chloromercuribenzoesäure wird gezeigt, daß dieses abnormale Hämoglobin ein Hämoglobin mit abnormaler β-Kette mit blockierter Cys-(β 93)SH-Gruppe ist. J. V. Dacie, A. J. Grimes, A. Meisler, L. Steingold, E. H. Hemsted, G. H. Beaven und J. C. White: Hereditary Heinz-body anaemia. A report of studies on five patients with mild anaemia. Brit. J. haemat. 10: 388–402 (1964). Es wird ein abnormes Hämoglobin durch Zonenelektrophorese im Stärkegel und im Agargel nachgewiesen. Dieses Hämoglobin unterscheidet sich vom normalen Hämoglobin auch durch Hitzelabilität. Ein dominanter Erbgang wird angenommen. M. Goudemand, G. Biserte, D. Habay und D. Vogel: Hémoglobine anormale et anémie hémolytique familiale avec inclusions érythrocytaires et urines noires. Nouv. Rev. franç. Hémat. 4: 487–504 (1964). Mit Fingerprint-Technik und Stärkegel-Elektrophorese wird ein abnormales Hämoglobin (abnormale Peptide) nachgewiesen. Der Erbgang dieser Erkrankung war dominant.

Auf Grund der bisher vorliegenden Untersuchungen ist daher als Ursache für dieses Krankheitsbild mit großer Wahrscheinlichkeit eine neue Hämoglobinopathie mit dominantem Erbgang anzunehmen.

sprechende Erniedrigung der Aktivität eine hämolytische Anämie zum Teil mit Innenkörperbildung verursacht werden kann, waren in den bisher untersuchten Fällen, ebenso wie in unserem Fall, normal. Die Erhöhung einiger Fermentaktivitäten entspricht lediglich dem allgemeinen Muster hämolytischer Anämien verschiedener Genese. Das gleiche gilt für die Bestimmung von Coproporphyrin, Protoporphyrin, Porphobilinogen und δ-Aminolävulinsäure.

Es ist bekannt, daß reduziertes Glutathion in den Erythrozyten die Bildung von Oxydationsprodukten des Hämoglobins (Methämoglobin, Sulfhämoglobin, Heinzsche Innenkörper) verhindert (3). In unserem Fall war Methämoglobin nicht nachweisbar, der Glutathiongehalt der Erythrozyten war normal, und auch beide GSSG-Reduktasen wiesen eine normale Aktivität auf. Eine besondere Exposition gegenüber oxydierenden Substanzen bestand nicht, ebensowenig konnte ein Mangel an Glukose-6-Phosphatdehydrogenase nachgewiesen werden.

Durch den beschriebenen Fall wird erneut die Bedeutung eines genetischen Defektes bei der Auslösung der Erkrankung unterstrichen. Die Tochter zeigt eine eindeutige, wenngleich bisher weitgehend kompensierte, hämolytische Anämie, wobei jedoch nur vereinzelte Erythrozyten die charakteristischen Innenkörper aufwiesen. Es kann demnach in Übereinstimmung mit der Beobachtung von Mozziconacci et al. (7) als sehr wahrscheinlich angenommen werden, daß die Innenkörper in großer Zahl erst nach der Splenektomie auftreten. Sie dürften bei den nicht splenektomierten Patienten in der Milz aus den Erythrozyten entfernt werden. An Hand der bisher beobachteten zwei Fälle mit gesichertem familiärem Vorkommen (9 eigene Beobachtung) kann ein dominanter nicht geschlechtsgebundener Erbgang angenommen werden. Es ist möglich, daß bei unserem Patienten der Gendefekt erstmalig aufgetreten ist, da von Vater und Mutter keinerlei Hämolysezeichen bekannt sind. Eine außereheliche Zeugung ist selbstverständlich nicht auszuschließen.

Für die Zukunft erscheint uns die klinische Erfassung der Patienten mit dieser laboratoriumsmäßig gut definierten hämolytischen Anämie wichtig, da sie, in Analogie zu anderen derartigen bekannten Krankheitsbildern, ein «Experiment der Natur» mit Ausfall eines Gens darstellen dürften. Es ist zu hoffen, daß an Hand weiterer Untersuchungen an diesen Patienten ein besserer Einblick in den Erythrozytenstoffwechsel gewonnen werden kann.

Zusammenfassung

Es wird der siebente Fall einer in den Laboratoriumsbefunden gut umschriebenen angeborenen hämolytischen Anämie mit massiver Mesobilifuscinurie und Auftreten zahlreicher Innenkörper nach Splenektomie mitgeteilt. Die Tochter des Patienten war eine knapp kompensierte hämolytische Anämie mit geringer Innenkörperbildung auf. Verschiedene Enzyme des Glucosestoffwechsels wiesen normale bzw dem Grad der Anämie entsprechende erhöhte Aktivitäten auf. Der Glutathionstoffwechsel war soweit untersucht, normal. Die Erythrocytenporphyrine bzw deren Vorstufen waren normal.

Summary

The seventh case is described of a form of haemolytic anaemia with characteristic laboratory findings massive mesobilifuscinuria and the presence of numerous inclusion bodies after splenectomy The patient' daughter presented barely compensated haemolytic anaemia with moderate inclusion body formation. The activity of various enzymes concerned with carbohydrate metabolism was either normal or slightly increased in proportion to the degree of anaemia. Glutathione metabolism as far as could be ascertained was normal. Red-cell porphyrins and porphyrin precursors were also normal.

Résumé

Description du septième cas d'une forme d'anémie hémolytique congénitale qui présente quelques traits caractéristiques relevés par les examens de laboratoire une mésobilifuscinurie massive et la présence de nombreuses inclusions érythrocytaires après la splénectomie. La fille du malade présente une anémie hémolytique à peine compensée avec une formation d'inclusions érythrocytaires modérée. Différents enzymes du métabolisme des hydrates de carbone présentent une activité légèrement augmentée selon le degré de l'anémie. Le métabolisme du glutathion est autant qu'il ait été étudié normal. Les porphyrines des érythrocytes et leurs précurseurs sont normaux.

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Varia

Heinz Karger Memorial Prize

The Dr. Heinz Karger Memorial Foundation in Basle announced last year an international competition for outstanding medical-scientific papers, to be written on the subject 'Enzymology of Leukemic Cells'. The Council of the Foundation—which comprises Professor H. LÜDIN, Basle, Professor G. MAYOR, Zürich, Dr. R. RICHTERICH, Berne, Professor J. R. RÜTTNER, Zürich, and the publisher Mr. T. KARGER—*has now* awarded the sF 3000.– prize to the two American research workers Dr. J. B. BLOCK and Dr. S. L. BONTING of the National Institutes of Health, Bethesda, Md., for their paper about 'ATPase Transport in Normal and Leukemic Leucocytes'.

This paper will be published in the international journal *Enzymologia biologica et clinica*.

Highly recommended were the papers submitted by Dr. W. WILMANNS from Tübingen (awarded the second prize) and Prof. D. DIOGUARDI of Cagliari and Prof. A. AGOSTONI of Milano (awarded the third prize).

The Council of the Foundation has also announced that the subject of the second competition, to be held next year, will be 'Microangiological Problems in Arteriosclerosis'.

From the University Medical Clinic, Freiburg im Breisgau
(Director: Prof. L. Heilmeyer)

Diagnosis of Iron Storage Diseases with Desferrioxamine (Desferal Test)

By Friedrich Wöhler

The cardinal symptom of iron storage disease in the human organism is as a rule hypersideraemia. To corroborate the diagnosis, however, it is necessary to demonstrate by histochemical methods that iron deposition in the tissues is increased. For this purpose, skin, biopsy liver puncture, or even splenic puncture is essential. Following the discovery of desferrioxamine, a complexing agent with a quite specific affinity for iron, it was an obvious step to see whether this compound could be used in the diagnosis of iron storage diseases. The studies conducted by Wöhler in 1961 (4) suggested that this would in fact the possible. It was found in these studies that the administration of desferrioxamine to normal subjects produces a rise in the serum iron owing to the removal of iron from endogenous iron-containing compounds. The iron thus mobilised then circulates in the blood in the form of a desferrioxamine iron complex until it is eliminated by the kidneys because of its low molecular weight of approx. 614. The subsequent therapeutic use of desferrioxamine in patients with iron storage diseases yielded very good results in haemochromatosis, generalised organ siderosis, liver cirrhosis, pulmonary haemosiderosis, sidero-achrestic anaemia, thalassaemia minor transfusion haemosiderosis, etc. (5 6)

The aim of our present investigation was to find out whether a single dose of desferrioxamine (500 mg intramuscularly or subcutaneously) would produce any regular changes in serum iron concentration and urinary iron excretion in a large number of normal subjects, as well as in patients with haemochromatosis, haemolytic anaemia, porphyria cutanea tarda, and other iron storage diseases, and in patients with infectious diseases. If the changes

produced by desferrioxamine were in fact found to follow a regular pattern, a simple test for the diagnosis of pathological iron deposition would then be possible.

Material and Methods

The normal subjects comprised 27 males and 15 females with no past history of disease. The 13 patients with haemochromatosis were divided up into an untreated group (7) and a group (6) which previously had been treated for short time. Also included in the study were 3 cases of porphyria cutanea tarda and massive iron storage, as well as 2 cases without massive iron storage in the liver cells. In addition, we investigated 1 case each of liver cirrhosis with siderosis, hepatitis, sickle-cell anaemia, thalassaemia, leukaemia, panmyelopathy with severe transfusion haemosiderosis, and tertian malaria, 8 cases of acute infection, and 10 cases in which an infectious disease had just been overcome and was no longer clinically manifest.

Desferrioxamine was administered to each patient in single intramuscular dose of 500 mg, using the commercially available ampoules of Desferal (Ciba). Subcutaneous administration would also have been possible because comparative studies yielded similar results.

The *serum iron* levels were measured using the method of HEILMEYER AND PLÖTNER (2).

Urinary iron excretion was determined as follows 8 drops of concentrated sulphuric acid 96% for analyses, 5 drops of trichloro-acetic acid 65% purest, and 2 drops of perchloric acid 70% for analyses were added to 0.5 ml urine in an iron-free test tube. The test tube was then heated over flame until the urine was largely ashed and had turned black. A further 5 drops of trichloroacetic acid 65% were then added until, after further ashing, only a water-clear drop was left in the test tube. After cooling, 1 ml of iron-free doubly distilled was pipetted into the test tube, followed by 1 drop of p-nitrophenol 1% as indicator 25% ammonia for analyses was then added drop by drop until the contents of the tube turned a light yellow colour whereupon 10% hydrochloric acid was added drop by drop until the contents became colourless. A further 3–6 drops of 10% ascorbic acid for analyses and 3–6 drops of 1% o-phenanthroline for analyses were then added. The contents of the tube were then titrated with 10% ammonia for analyses and 10% hydrochloric acid for analyses until a maximum red colour developed. The red colour was then measured with an S 49 filter in a Zeiss Elko III. The layer in the measuring cell was 0.5 cm thick. The iron content was calculated with the aid of the equation

$$\frac{\text{extinction} \times \text{end volume} \times 2 \times 5 \text{ (o-phenanthroline factor)}}{0.5 \text{ (thickness of layer)}}$$

— blank value = iron in μg/ml.

The blank value was determined by ashing iron-free doubly distilled water instead of urine. To calculate the total iron excreted in 24 6, or 3 hours, the iron content in μg/ml was multiplied by the volume of urine excreted in the relevant period. The results were expressed in mg% or in mg total iron. Total iron-binding capacity was ascertained by RAMSAY' method (3) Determinations of desferrioxamine in the urine were kindly carried out by Dr KEBERLE AND Dr FITZGERALD of Ciba Ltd., Basle, and we should like to thank for their assistance. Hb was measured routinely with calibrated Zeiss haemometer

Procedure. To ensure that reliable baseline values were obtained for the urinary iron concentration in normal subjects, all members of the control groups were given 400 ml

of tea to drink on the day before the actual test and their urine was then collected over period of 6 hours. All test subjects had of course to void their bladders before the start of the test. In the pathological cases w deliberately omitted the preliminary test, firstly to void imposing an unnecessary strain on the patients, and secondly to ensure that the conditions for the test would resemble as closely as possible those encountered in practice. It is advisable, however—and this we did—to obtain specimen of urine prior to the test, to determine its iron content in mg% and to use this value as baseline. In addition, the haemoglobin was determined and sample of blood taken for measurement of the serum iron just prior to the test. 500 mg desferrioxamine was then injected intramuscularly after which the patients were given 400 ml tea to drink so as to ensure reliable and reasonably copious excretion of urine. Further blood samples were taken for serum iron determinations 2 and 4 hours after the injection. Total iron-binding capacity was determined in the blood serum prior to the test. In practice, this determination can be omitted. Urine was collected for period of 6 hours starting from the time of injection. In some of our test subjects and patients, the urine was collected in two separate portions 3 and 6 hours after the injection and its iron content determined. This was done in order to establish the period of maximum iron excretion. The urine was collected in iron-free plastic containers on glacial acetic acid (5 ml) so as to avoid precipitation, because it had been found that up to 25% of the iron excreted may be present in the sediment. All the substances used came from Merck, Darmstadt.

Statistics: The values obtained for the serum iron and urinary iron were submitted to statistical analysis, the variation of the normal values being calculated with the aid of Geigy's scientific tables. Only the results outside the 2σ limit were considered as significant. (A detailed account of the methods employed and of the statistical procedure will be found in the inaugural dissertation of H. Koch, Freiburg, 1964.)

Results

To obtain reliable baseline values, the serum iron level was determined in the *normal tests subjects* (27 males and 15 females) prior to the intramuscular injection of 500 mg desferrioxamine, as well as 2 and 4 hours afterwards. As figs. 1 and 2 show the serum iron invariably showed a significant increase within 2 hours.

For instance, in the male subjects it rose from mean baseline value of 114.6 µg% to 153.9 µg% after 2 hours, and then, after further 2 hours, fell again to 99 µg%, i.e. to below the mean baseline value. In the female test subjects, in whom the baseline value of 95.2 µg% (mean value) was lower than in the males, the rise after 2 hours was also less marked (to 133.3 µg%), and the level then decreased after 4 hours to mean value of 92.5 µg%, i.e. approximately to the baseline value. This finding, however, is subject to certain reservations because for technical reasons no values were obtained 4 hours after the injection in 8 subjects. As regards the 7 subjects in which the values were obtained, the level dropped considerably below the baseline in cases 2, 12, and 13. The haemoglobin values, like the serum iron levels, were lower in the female group than in the males, but were for all practical purposes within the normal range. Determination of total iron-binding capacity in the test subjects yielded roughly normal results. The mean values for iron excretion measured over period of 6 hours on the day prior to the actual test—i.e. *without* desferrioxamine—were approximately the same in both sexes, being 0.028 mg total urinary iron in the males, and 0.024 mg% or 0.068 mg total urinary iron in the females. Three hours after the injection of 500 mg desferrioxamine iron excretion was considerably increased in both test groups. Owing to the small number of

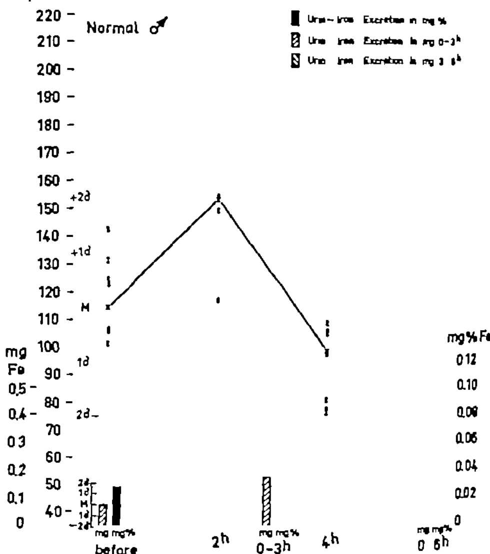


Fig. 1 Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in 27 normal male test subjects. Mean baseline value: 114.64 (n = 27). Mean value after 2 hrs 153.87 (n = 20). Mean value after 4 hrs 99.0 (n = 16)

subjects involved, however the figures obtained possess only an indicative value. In 8 males excretion amounted to 0.074mg% or 0.174 mg total urinary iron in 3 hours. In the 3 females studied, the mean values were 0.055 mg% and 0.179 mg total urinary iron.

Of greater importance, however are the figures for the entire 6-hour period following the injection. The mean values for this period in the 27 male subjects were

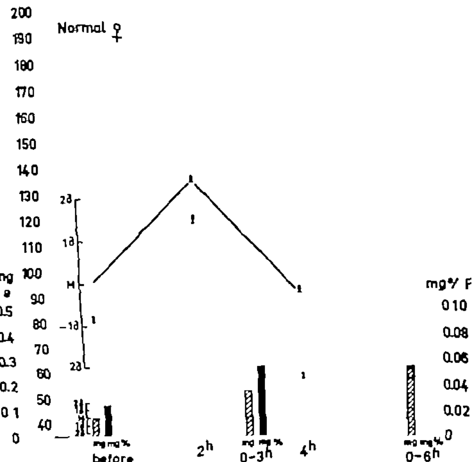


Fig. 2. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in 15 normal female test subjects. Mean baseline value 95.25 (= 15). Mean value after 2 hrs 135.24 (n = 12). Mean value after 4 hrs 93.94 (= 7)

0.113 mg% and 0.398 mg total urinary iron. Hence, urinary iron excretion, normally very low had increased significantly (excretion of total urinary iron was more than 5 times the normal value). This increase in iron excretion was also encountered in the female subjects, although it is interesting to note that the values obtained in this group were *not so high, the mean figures for the 15 females being 0.079 mg%* and 0.264 mg total urinary iron. These also indicate the physiological differences between the two sexes in respect of iron metabolism, i.e. they show that the iron deposits are relatively smaller in women than in men owing to the physiological loss of blood during menstruation. These differences are also reflected by the serum iron pattern and, to lesser extent, by the haemoglobin, which is likewise lower in women than in men.

To sum up these results in normal subjects display a regular pattern in respect of the changes in serum iron level and urinary iron excretion following a single dose of desferrioxamine. It may therefore be assumed that in normal subjects, too, desferrioxamine is able to remove iron ions chiefly from the ferritin and haemosiderin deposits the circulation of the resultant desferrioxamine iron complex in the blood raises the serum iron level, and the excretion of the complex provokes an increase in the urinary iron content over a certain period of time. The period of 6 hours was chosen arbitrarily it is unlikely that all the desferrioxamine-iron complex has been excreted by the end of this time, but it seemed better for practical reasons to set a time limit for a test procedure. That excretion of the complex has not in fact been completed by the end of 6 hours can be inferred from determination of the desferrioxamine recovered unchanged—i. e. not loaded with iron—in the urine. Total desferrioxamine excretion over the 6-hour period in the two groups was 100 and 115 mg respectively The most important finding obtained from these tests, however is that the amount of iron which can be removed by desferrioxamine is not apparently unlimited in the normal test subject, but is kept within relatively narrow boundaries. Theoretically 500 mg desferrioxamine would be capable of binding some 45 mg iron, but in our tests only 0.398 mg iron on the average appeared in the urine over the 6-hour period. This finding is not invalidated either by the fact that in certain cases up to 181 mg desferrioxamine (average value approx. 115 mg) was recovered in the urine. At least 300 mg desferrioxamine must therefore have been available to take up iron, and this amount would have been capable of binding a maximum of 27 mg That, on the other hand, a very large proportion of the mobilised iron was excreted in these 6 hours is apparent from the decrease in the serum iron level by the end of this period, and also from our earlier studies in which we obtained in normal subjects a maximum total urinary iron excretion of some 1.2 mg (minimum 0.3 mg) over a period of 24 hours following a single dose of desferrioxamine. If desferrioxamine is administered daily to normal subjects, however a rise in the daily urinary iron excretion to a maximum of 3 7 mg can be observed up to about the 4th day

The results obtained in normal test subjects, which indicate that the serum iron concentration followed a regular pattern and that relatively large amounts of iron were excreted in the urine over

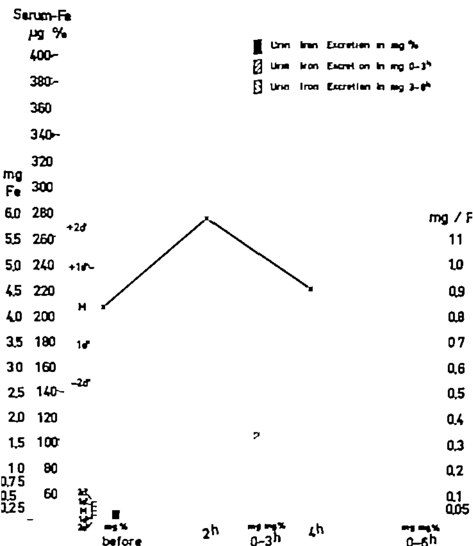


Fig. 3. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in untreated haemochromatosis patients.

a period of 6 hours, can be regarded as normal values. The results obtained in the patients with confirmed iron storage diseases, as described below differed significantly from these normal values.

The pattern of the *serum iron concentration and urinary iron excretion in patients with haemochromatosis* is reproduced in figs. 3 and 4. In all

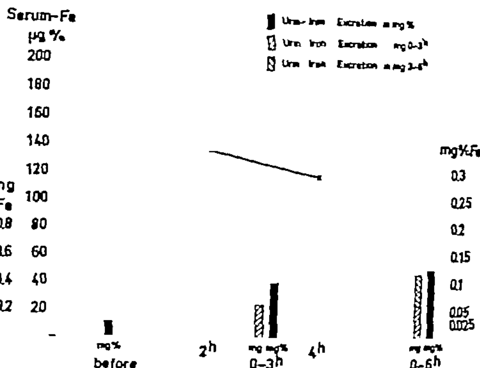


Fig. 6. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in patients with porphyria cutanea tarda without by any appreciable iron deposition.

In the *patients who had been under treatment for a short time* (fig. 4) the results were roughly similar i.e. the serum iron level showed a typical rise from a mean value of 162 μg% to 213.3 μg / after 2 hours and to 233.6 μg / after 4 hours. The lower baseline value in these patients reflects the success of desferrioxamine treatment, for as fig. 3 shows, the serum iron levels in untreated patients were higher. The total urinary iron excretion (mean value of 4.116 mg) was as high as in the untreated patients, as was to be expected. The amount of iron excreted in the first 3 hours was lower than the amount excreted in the second 3 hours. It seems that a certain length of time must elapse before the desferrioxamine molecules are saturated with iron to their maximum capacity and are then excreted in the form of ferrioxamine.

The iron excretion of patients with *porphyria cutanea tarda* was studied in further tests. On the basis of the results obtained from histochemical examination of liver biopsy material, we divided

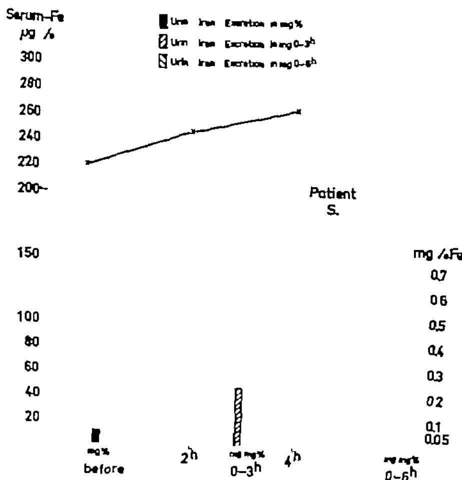


Fig. 7 Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in patient with panmyelopathy and transfusion siderosis.

these cases into those with iron storage in the liver cells and those without. As can be seen from fig. 5 the rise in serum iron after 2 hours displays the pattern typical of severe haemochromatosis. A decrease in the serum iron level was observed after 4 hours in only one case. Total urinary iron excretion (1.5 mg) was less than in the haemochromatosis patients, but significantly increased in comparison with the normal values. On the other hand, both the serum iron levels and the urinary iron excretion could still be

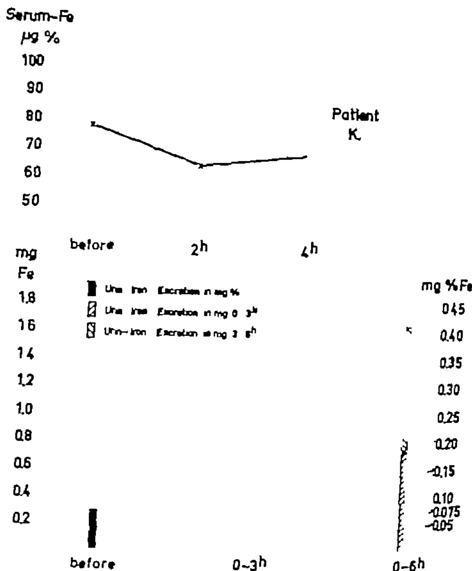


Fig. 4. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in woman with sickle-cell anaemia.

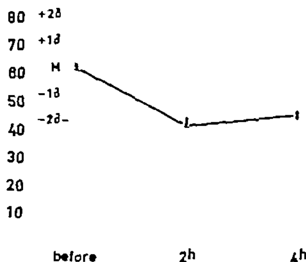
regarded as normal in the patients illustrated in fig. 6. Moreover an increase in iron excretion was not to be expected in these patients in the light of the histochemical findings, which indicated only slightly increased iron deposits in the liver parenchyma.

Single cases of *various other diseases* such as sickle-cell anaemia, sidero-achrestic anaemia, liver cirrhosis with siderosis, and pan-

myelopathy with transfusion haemosiderosis, were also studied. As was to be expected, these patients (fig 7) who had high serum iron baseline values, displayed an increase in serum iron levels for up to 4 hours after the injection and a marked increase in urinary iron excretion (3.3 mg in 6 hours) It is interesting to note that urinary iron excretion was increased (1 65 mg) even in a case of sickle-cell anaemia (fig 8) in which the serum iron level was relatively low and decreased in response to desferrioxamine. In this patient, too, therefore, siderosis would seem to have developed as a result of haemolysis. This finding that haemolysis iron is mobilised appears important to us, inasmuch as it indicates the possibility of employing desferrioxamine treatment to improve states of pathological iron deposition in the large number of patients with sickle-cell anaemia and siderosis. In one case of liver cirrhosis with siderosis an increase in urinary iron excretion was also observed (1.359 mg) as was likewise to be expected. It may also be of interest to point out that in one patient with hepatitis, who displayed a high serum iron baseline value of 214.5 µg /‰ urinary iron excretion was approximately doubled. In one patient with acute myelosis, who, however had been given several transfusions, no difinite change occurred in the serum iron levels following the injection of desferrioxamine, but urinary iron excretion was increased (0 867 mg) One patient with sidero-achrestic anaemia displayed, as expected, an increase in urinary iron excretion (2.29 mg within 6 hours) Here, too, as in the haemochromatous patients, the greatest amount of iron was apparently excreted between the third and the sixth hour

To sum up, it may be said that the patients with confirmed siderosis (liver biopsy etc.) regularly exhibited—apart from exceptions mentioned—a typical rise in the serum iron level and an increase in urinary iron excretion. It thus appears justified to regard a significant increase in iron excretion following the injection of desferrioxamine as a sign of iron storage disease in the organism. Depending on the clinical findings, such cases could then possibly be treated with desferrioxamine.

It was of particular interest in these studies to test the response to desferrioxamine in patients with *infectious diseases* since it is known that iron ions are stored in the R.E.S. during disorders (1) Fig. 9 illustrates the response in acute infectious diseases. In all these patients, the serum iron level prior to the administration of desferrioxamine was markedly reduced owing to the infection. In



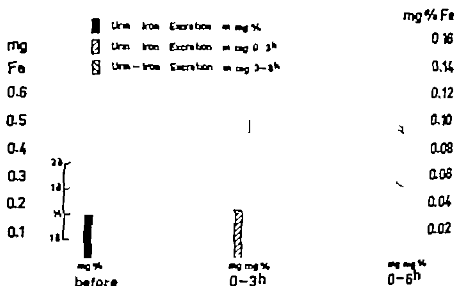


Fig 9. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in patients with acute infectious diseases and markedly reduced serum iron concentrations.

response to desferrioxamine an additional more or less pronounced decrease occurred, the baseline value being reduced on the average by about onethird to 42.2 µg %. After 4 hours the value was still roughly as low in other words, depression of the serum iron levels

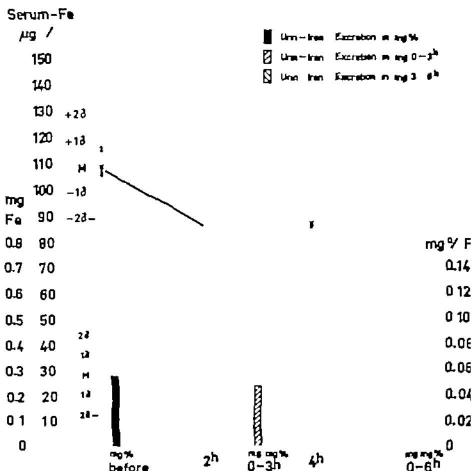


Fig. 10. Serum iron level and urinary iron excretion in response to an intramuscular injection of 500 mg desferrioxamine in patients who no longer had any clinical manifestations of an infectious disease and whose serum iron concentration was normal.

persisted. The amount of iron excreted, however averaged 0.5 mg total urinary iron and was therefore a little higher than the normal value. Hence, although desferrioxamine is apparently able to eliminate iron in patients with infectious diseases, it does not cause the typical rise in serum iron levels encountered, for instance, in normal subjects. On the contrary in view of the decrease in the serum iron levels, it may be presumed that desferrioxamine tends to take up iron ions from the labile pool and mobilises less iron from the RES-cells than it does in normal subjects. During an infection the iron ions

appear to be much more firmly fixed than under normal conditions. Fig 10 illustrates the situation observed in patients who no longer have any clinical manifestations of an infectious disease and whose serum iron levels are normal. Surprisingly enough, the test revealed that although the baseline values were within the normal range, desferrioxamine provoked not an increase but a decrease in the serum iron levels. *Admittedly* the decreases were not so considerable as in patients with frank infections, but they were still clear-cut. The total urinary iron excretion was somewhat higher than in normal subjects. The results obtained illustrate that in patients with infectious diseases, too the iron levels seem to display a regular pattern in response to desferrioxamine, a pattern which is marked by a decrease in the serum iron levels associated with a slight increase in total urinary iron excretion.

Summary

The intramuscular administration of desferrioxamine produces a regular increase in serum iron levels and of urinary iron excretion in normal subjects. These changes are due to the fact that desferrioxamine mobilises iron from the iron deposits in the organism and binds it into a complex which circulates in the blood plasma and is then excreted via the kidneys. The mean value for total urinary iron excretion after 6 hours following an intramuscular dose of 500 mg desferrioxamine was used as basis for test designed to detect pathological iron deposition in the organism. In cases of haemochromatosis, transfusion haemosiderosis, sidero-achrestic anaemia, liver cirrhosis with siderosis, sickle-cell anaemia, and also porphyria cutanea tarda, urinary iron excretion was found to be significantly increased. This test constitutes an important addition to the diagnosis of iron storage diseases. In infectious diseases, no increase in the serum iron level occurs, while total urinary iron excretion remains roughly within normal limits.

Résumé

L administration intramusculaire de desferrioxamine produit chez le sujet normal régulièrement une augmentation du taux *du fer sérique* et de *l'excrétion du fer* dans les urines. Ces changements sont dus au fait que la desferrioxamine mobilise le fer de ses dépôts dans l'organisme et le fixe à un complexe qui circule dans le plasma sanguin et est excrété par les reins. La quantité moyenne de fer excrétée dans les urines durant les 6 heures qui suivent l'injection intramusculaire d'une dose de 500 mg de desferrioxamine sert de base à un test destiné à la détection de dépôts pathologiques de fer dans l'organisme. Dans les cas d'hémochromatose, d'hémosidérose à la suite de transfusions sanguines répétées, d anémie sidero-achrestique, de cirrhose d foie ec sidérose, d'anémie à hématies falciformes et aussi de porphyrie cutanée tardive, l' de fer dans les urines est augmentée de façon significativ e. Ce test pporte contribution au diagnostic des thésaurismoses du fer. Dans les maladi ne sérique ne élève pas, l'excrétion urinaire totale de fer normales.

Zusammenfassung

Intramuskulär verabreichtes Desferrioxamin führt bei gesunden Versuchspersonen zu einem gesetzmäßigen Serumeisenanstieg und zu einer vermehrten Urineisenausscheidung. Diese Veränderungen werden bedingt durch Mobilisation von Eisen aus den Depots des Organismus, den dann kreisenden Desferrioxamineisenkomplex im Blutplasma und seine Ausscheidung durch die Niere. Da sich für die Gesamturineisenausscheidung nach 6 Stunden bei Gabe von 500 mg Desferrioxamin i. m. ein Mittelwert ergab, war es möglich, diesen als Grundlage für einen Test zur Erkennung von Eisenspeicherungen im Organismus zu benutzen. Hämochromatosen, Transfusionshämosiderosen, sideroachrestische Anämien, Leberzirrhosen mit Siderose, Sichelzellenanämie, aber auch Fälle von Porphyria cutanea tarda ergaben signifikant erhöhte Urineisenausscheidungen. Dieser Test stellt eine wichtige Bereicherung der Diagnostik von Eisenspeicherkrankheiten dar. Von großer Wichtigkeit erscheint, daß bei Infekten nach Desferrioxamingabe ein Abfall des Serumeisens gesetzmäßig eintritt; dieser ist auch bei klinisch nicht mehr manifesten Infekten mit normalem Serumeisenspiegel zu beobachten. Die Gesamturineisenausscheidung ist dabei geringgradig vermehrt.

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Acta haemat. 32: 338–347 (1964)

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Tolerance to Phenindione of Patients with Different Thromboembolic Disorders

By F. Nour Eldin and F. J. W. Lewis

Unexpected temporary variations in the prothrombin time occur during coumarin therapy. These have been generally thought to be due to changes in the patient's physical activity and/or drugs and food intake which may directly or indirectly affect the metabolism of coumarin. Variations in the requirements of anticoagulant drugs may arise as a result of administering other drugs. The action of vitamin K and salicylates has been known for decades while that of antibiotics (5), d-thyroxine (14) and Atromid (12) has recently been described. Hitherto, however, no relation between the site of the thrombo-embolic lesions and the tolerance to these drugs, has been reported.

In the present paper, we demonstrate varied prothrombin time responses to phenindione of patients with different clinico-pathological conditions.

Methods

Citrated blood: 9 volumes of blood were mixed with 1 volume of 3.8% trisodium citrate.

Brain extract. T 0.3 g of acetone-dried human brain tissue was added 10 ml 0.5% phenol in 0.9% NaCl. This was incubated at 37 °C for 20 minutes, stirring gently every 2—3 minutes. By leaving the suspension undisturbed for further 5 minutes, the large particles sedimented and the supernatant fluid was removed for use.

Prothrombin time: 0.025 M $CaCl_2$ was added to equal volumes of citrated blood and brain extract at 37 °C, recording the clotting time. Normal controls gave prothrombin times of 12 ± 1 sec.

Results

Response curves. Whilst assessing the therapeutic benefit derived from coumarin therapy in different diseases, it was noted that on drawing the dosage and the prothrombin time on the scale shown

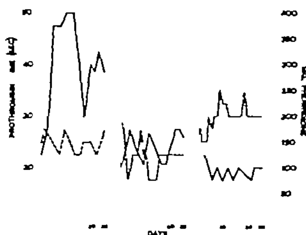


Fig 1 Response curves encountered in patients receiving Phenindione therapy Prothrombin-time curve (A) above, (B) intersecting, and (C) below the dosage curve (———).

in fig 1 3 types of curves (termed hereafter response curves) could be identified A) Where the prothrombin time curve is in the greater part much higher than the phenindione dosage curve. B) the 2 curves are intersecting and C) the prothrombin time curve is almost always below that of the drug This prompted further investigations.

Table I
Period of phenindione therapy

Disease	Days up to 10	20	30	40	50	> 50
Heart conditions	5	3	14	20	7	23
Pulmonary infarction	6	10	5	4	1	1
Venous thrombosis	15	20	5	4	1	0
Arterial occlusion	0	4	2	0	1	1
Cerebral infarction	4	1	3	0	0	0
Mesenteric embolism	1	0	1	0	0	0

Distribution of curves The patients investigated consisted of the 167 patients given anticoagulant therapy during 1961 in Southmead Hospital. Of these, 3 cases received heparin only and 4 cases were given drugs other than phenindione and accordingly were not included in the present study Table I gives the period during which

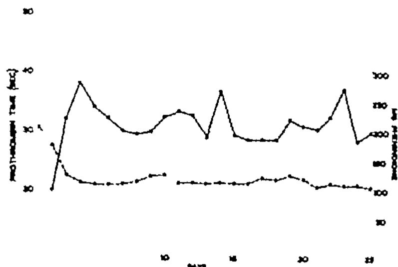


Fig. 2. Prothrombin-time response (———) to Phenindione (•—•--•) of patients with myocardial infarction

Table II
Frequency of prothrombin time curves in different diseases.

Condition	Total	Curve (fig. 1) A	B	C
Myocardial infarction	60	39 (65%)	19 (32%)[2]	2 (3%)
Heart failure*	7	7 (100%)	0	0
Pulmonary infarction	21	16 (76%)	4 (19%)	1 (5%)
Venous thrombosis	28	6 (21%)	14 (50%)[3]	8 (29%)
Arterial occlusion	8	4 (50%)	4 (50%)	0
Cerebral thrombosis	4	2 (50%)	2 (50%)	0

due to hypertension (2), toxaemia (1) myocarditis (1) and valvular defects (3)
3 cases had diabetes
one diabetic patient and one case with porphyrinuria.

the remaining 160 patients received phenindione. The prothrombin-time response to this drug in each of the 128 cases who received therapy for a period of more than 10 days were then analysed. The number of patients and the distribution of the response curves (described above) for each disease is shown in table II. From this it will be seen that the patients with venous thrombosis have a different distribution. When a single graph was constructed for each group over a period of 25 days, very similar responses were found in cases of myocardial infarction and pulmonary embolism (fig 2 and 3) The patients with venous thrombosis had a different curve (fig 4)

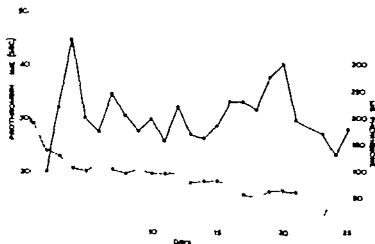


Fig. 3. The effect of Phenindione (•—•—•) on prothrombin-time (———) of patients with pulmonary infarction.

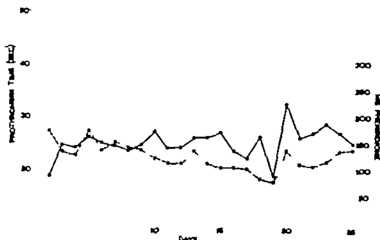


Fig. 4. Prothrombin-time changes (———) during Phenindione (•—•—•) therapy in cases of venous thrombosis.

Effect of different factors. Possible causes for this apparent difference in tolerance to phenindione were considered as follows:

1. Age and Sex. Venous thrombosis being commoner in females and myocardial infarction occurring more frequently in males suggested that sex might contribute to this picture. *However* 12 cases of the 21 patients with pulmonary embolism were females

Table III
Age distribution.

Age	Myocardial Infarction	Pulmonary embolism	Venous thrombosis
11—20	0	0	1
21—30	0	0	2
31—40	3	1	5
41—50	12	2	6
51—60	26	8	10
61—70	19	8	2
70	0	2	2

Table IV
Prothrombin curves in cases receiving antibiotics.

Condition	Antibiotic	Cases	Prothrombin curve[1]
Myocardial infarction	Crystamycin	2	A, B
	Penicillin	2	A, B
	Tetracyclin	2	B
Pulmonary infarction	Penicillin	2	A, B
	Tetracyclin	6	4 A, 2 B
Venous thrombosis	Crystamycin	1	C
	Tetracyclin	2	C

A mixture of penicillin, streptomycin and dihydrostreptomycin (Glaxo)
see fig. 1

and the 5 females with heart failure (due to causes other than coronary disease) all gave curves type A. This would appear to exclude sex from being the main factor. Similarly the age distribution of the patients analysed (table III) seems to be of no particular importance.

2. Drugs. Heparin was administered to 40, 52, and 39 % of our patients with myocardial infarction, pulmonary embolism and venous thrombosis respectively. Its administration did not appear to influence the type of curve obtained.

Barbiturates having been given to 48, 40, 52 % of cases of myocardial infarction, pulmonary embolism and venous thrombosis respectively does not appear to affect the sensitivity to Phenindione. The cases receiving other sedatives and tranquillisers in all 3 groups were too few to assess.

The similarity between the curves of patients with coronary disease, 20 of whom received glycerine trinitrate and those of cases with pulmonary infarction, none of whom received this drug would

suggest that the response curve is not influenced by this therapy. Furthermore no correlation was found between the administration of this compound and the type of curve in the former group.

Antibiotics were administered for a period of 4 days or more to 6, 8 and 3 patients with heart conditions, pulmonary lesions and venous thrombosis respectively. From the analysis presented in table IV although neither erystamycin nor tetracycline appear to have significant influence on the tolerance to phenindione (prothrombin curves type C being found in the 3 cases with venous thrombosis receiving these antibiotics) the number of cases is considered too small to be significant.

Diuretics were not administered to patients with venous thrombosis but were used in 7 cases of coronary disease and 3 cases of pulmonary embolism. Although 6 of the former group and all the latter cases had type A response curves, the small number of the cases makes it difficult to decide whether or not these drugs per se affect the action of coumarin drugs on the blood-clotting mechanism. However the possible effect of the underlying pathological condition, for which diuretics were prescribed, is referred to in the discussion.

Table V
Cases of venous thrombosis.

Precipitating condition	Total	Prothrombin curve*		
		A	B	C
Post-operative	12	3	5	4
Post-partum	6	1	3	2
Varicose veins	3	0	2	1
Unknown	7	2	4	1

* see fig. 1

Causes of venous thrombosis. Table V summarizes the range of conditions from which our cases of venous thrombosis have arisen. None can be incriminated for the variation in response within this group of patients. It would appear that the most likely explanation is the presence of certain overlapping in the individual behaviour which, apparently has no significant influence on the response of the group as a whole (figs. 2—4).

Relation between prothrombin-time and bleeding. Out of 4818 prothrombin time estimations, 876 were above 36 seconds (normal 11—12 seconds). Details of these are given in table VI. In spite of

thus, bleeding episodes occurred only on 21 occasions in 19 patients as demonstrated in table VII On many of these occasions, the prothrombin time was within the therapeutic range for coumarin drugs. Accordingly it is concluded that this blood clotting test, although offering a valuable guide for routine control of coumarin administration does not provide an absolute demarcation line for anticipating bleeding manifestations no bleeding being encountered in many patients with very prolonged prothrombin times while others suffer this complication despite a reasonable prothrombin time. Table VII also shows that the type of complicating haemorrhage bears no relation to the prothrombin time or the site of the thromboembolic lesion.

Table VI

Prothrombin times outside therapeutic range.

Prothrombin time (sec.)	Specimens
37—40	159
41—50	415
51—60	166
61—70	54
71—80	31
81—120	36
121—180	5
> 180	10
Total	876

Table VII

Bleeding during phenindione therapy

Type of Bleeding	Occasions	Prothrombin time, sec.	Cases affected
Haematuria	1	18	Venous thrombosis
	9	31—40	Myocardial 8[1] Pulmonary 1
	2	45+47	Pulmonary
	1	149	Venous
Haematoma	2	21+25	Myocardial and arterial
(subcutaneous +	4	31—40	Myocardial 3; Venous 1
intramuscular)	1	58	Pulmonary
Epistaxis	2	42+48	Myocardial
Melaena	1	22	Pulmonary
Cerebral	1	31	Myocardial

Normal 11—12 secs.

One case had also pyrexia and rash on back and abdomen.

Discussion

Without prejudice to the effect of coumarin drugs on the prognosis in thromboembolic disease (an aspect which is outside the scope of this paper) the present work demonstrates the presence of different prothrombin-time response to phenindione in different thromboembolic conditions. Compared with patients with venous thrombosis, cases of pulmonary embolism and cardiac infarction seem to have decreased tolerance to phenindione. Factors which could possibly contribute to this were examined. Neither sex nor any of the drugs administered could be incriminated. The inert action of barbiturates in this respect is in conformity with the work of Moreno and Gracia (7) who concluded that these compounds do not alter the tolerance to coumarin drugs. Antibiotics may alter the intestinal flora (15) and thus are liable to interfere with the production of vitamin K which ordinarily may act as a buffer against the coumarin drugs (8) This would decrease the tolerance (increased prothrombin times) to coumarin drugs. This was found to be the case by Magid (5) when receiving chloramphenicol, chlor tetracycline, neomycin—bacitracin, streptomycin—penicillin, but not with penicillin alone. The response of our cases appears not to be influenced by crystamycin or tetracycline.

The liver is one of the important organs concerned with the metabolism of vitamin K and the synthesis of prothrombin and other blood clotting factors (6, 2, 1) In cases with heart failure it is recognized that there is some disturbance of liver function. This may affect prothrombin production and/or render the liver more sensitive to coumarin drugs, and could account for the decreased tolerance in cases of cardiac and pulmonary infarction. That this is the most probable explanation is supported by the fact that prothrombin-time curve type A was observed in all 7 cases of heart failure due to causes other than coronary disease (table II) Renal insufficiency in these cases (13) possibly by decreasing their excretion may also lead to the apparent increase in the sensitivity to coumarin drugs.

None of the methods used for the estimation of prothrombin time, whether utilizing human or bovine brain (9 10) provides absolute criteria as to the occurrence of bleeding manifestations. The absence of bleeding in a patient with prolonged prothrombin

time indirectly supports the presence of differences between the in vivo and in vitro behaviour of clotting factors (4, 11).

Acknowledgments: We are grateful to Dr. H. G. Mather for reading the manuscript. We also thank our medical colleagues and members of the Records Department for access to the patients' notes, and the Ethel Showering Fund for defraying some of the expenses.

Summary

The prothrombin-time responses in 160 consecutive cases admitted to Southmead Hospital, who received phenindione for different thromboembolic disorders, have been analysed. Tolerance to this drug was lower in patients with cardiac infarction and pulmonary embolism than in cases of venous thrombosis. Possible factors contributing to this behaviour are examined. The limitations of prothrombin time estimations in the control of coumarin therapy are discussed.

Résumé

Chez 160 malades admis consécutivement à l'Hôpital de Southmead et qui recevaient du Phenindion pour différentes maladies thrombo-emboliques l'effet de ce médicament sur le temps de prothrombine a été étudié. La tolérance à ce médicament était moindre chez les malades ayant eu un infarctus du myocarde ou une embolie aux poumons que dans les cas de thromboses veineuses. Les facteurs possibles déterminant un tel comportement sont examinés. Les limitations des estimations du temps de prothrombine dans le contrôle de la thérapie aux coumarines sont discutées.

Zusammenfassung

Bei 160 nacheinander ins Southmead-Spital eingewiesenen Patienten, die wegen verschiedener thromboembolischer Krankheiten Phenindion erhielten, wurde die Wirkung auf die Prothrombinzeit untersucht. Die Toleranz für dieses Medikament war bei Fällen von Herzinfarkt und Lungenembolie geringer als bei Patienten mit Venenthrombosen. Die möglichen Ursachen dieses Verhaltens werden untersucht. Die Grenzen der Prothrombinzeitbestimmung bei der Kontrolle der Cumarinbehandlung werden diskutiert.

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Acta haemat. 32, 348–354 (1964).

Aus der I. Medizinischen Klinik und Poliklinik der Johannes-Gutenberg-Universität Mainz (Direktor: Prof. Dr. R. Duesberg)

Die Bedeutung der unveresterten Fettsäuren für die Resistenz von Erythrozyten

Von A. M. Ehrly, F. Gramlich und H. E. Müller

In einer früheren Arbeit (1) haben wir über die Erhöhung der osmotischen Resistenz von Erythrozyten durch Zusätze von unveresterten Fettsäuren berichtet. Diese Versuche mit ^{131}Jod-Ölsäure ergaben eine konzentrationsabhängige Verminderung der Hämolyse gegenüber hypotonen NaCl-Lösungen. Es wurde auch nachgewiesen, daß der Austausch markierter Ölsäure zwischen Plasma und Erythrozyten sehr schnell vor sich geht und daß eine besondere Affinität der unveresterten, freien Fettsäuren (UFS) zu den Erythrozytenproteinen gegenüber den Plasmaproteinen nicht vorzuliegen scheint. Eine selektive Anreicherung der UFS am Erythrozytenstroma war nicht zu erkennen. In Fortführung dieser Arbeit wurde die Wirkung von physiologisch vorkommenden UFS und von Substanzen untersucht, die analoge physikochemische Eigenschaften wie die UFS aufweisen. Zunächst soll die Wirkung von Ölsäure auf Blut besprochen werden. Daran schließen sich Untersuchungen an über andere physiologisch vorkommende UFS wie Linolsäure, Linolensäure und Arachidonsäure, weiterhin von Neutralfetten und schließlich von Substanzen, die ähnlich wie Fettsäuren zur Mizellbildung befähigt sind. Außerdem wurde als oberflächenaktiver Stoff das Digitonin untersucht. So konnte durch einen Vergleich der verschiedenen Stoffe die spezifische Einwirkung der UFS auf die Erythrozyten verdeutlicht werden.

Material und Methode

Blut gesunder Versuchspersonen wurde mit verschiedenen Antikoagulantien (Natrium-Oxalat, -Citrat und Heparin) ungerinnbar gemacht. Fettsäuren und andere Substanzen wurden nach einem bereits früher beschriebenen Verfahren (1) mit dem Blut vermischt.

Zur Prüfung der osmotischen Resistenz wurden Erythrocyten konventionell fallenden NaCl-Konzentrationen (0,6 bis 0,1%) ausgesetzt. Die mechanische Resistenz und die Inkubationshämolyse wurden nach Literaturangaben durchgeführt (2, 3). Der Hämatokrit wurde nach Wintrobe bestimmt.

Folgende Substanzen wurden verwendet: Ölsäure reinst der Firma Merck, Darmstadt, Linolsäure, Linolensäure und Arachidonsäure der Firma Roth, Karlsruhe, essentielle Phospholipoide der Firma Nattermann, Köln, Dodecylsulfonat der Firma Henkel, Düsseldorf, Cetyltrimethylammoniumbromid der Firma Schuchardt, München, Natrium-Oleat der Firma Riedel de Haen, Hannover.

Ergebnisse

a) Die osmotische und mechanische Resistenz von Erythrocyten nach Zugabe von Ölsäure bzw. Natrium-Oleat. Steigende Konzentrationen von Natrium-Oleat im Blut normaler Versuchspersonen verbessern die osmotische Resistenz beträchtlich (Abb. 1). Dabei wird die typische S-förmige Verlaufsform der Hämolyse bis zu einer Konzentration von etwa 90 mg% Natrium-Oleat nicht verändert. Bei

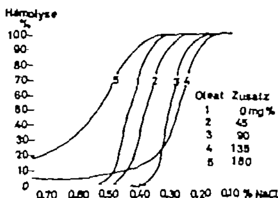


Abb. 1 Osmotische Resistenz von Erythrocyten in Abhängigkeit von der Konzentration an Natrium-Oleat.

dieser Konzentration ist im Gegensatz zum nicht behandelten Blut die 50% Hämolyse erst bei einer 0,3% NaCl Lösung (Normalwert 0,45% NaCl) erreicht. Bei höheren Dosen zugesetzten Oleats verschiebt sich zwar der Wert für die 50% Hämolyse noch weiter zur hypotonen Seite, doch besteht jetzt eine sichtbare Spontanhämolyse, die sich in einem abgeflachten unteren Bogen der Hämolysekurve («Schwanzbildung») ausdrückt. Noch höhere Konzentrationen führen zur totalen Hämolyse.

Sowohl Natrium-Oleat wie Ölsäure zeigten prinzipiell die gleichen Effekte. Eine Verschiebung des pH Wertes des untersuchten Blutes trat bei den verwendeten Konzentrationen an Ölsäure bzw Natrium-Oleat nicht ein. Ändert man allerdings den pH Wert des Blutes mit Na OH oder HCl, dann zeigt sich, daß nur das Oleat als negativ geladenes Ion die beobachtete Wirkung entfaltet, denn in alkalischem Milieu (pH 8) erhöht sich und in saurem Milieu (pH 6) verringert sich bei gegebener Konzentration die Wirkung der Ölsäure auf die osmotische Resistenz der Erythrozyten. Es ist also dem Oleat Ion und nicht der undissoziierten Säure die Wirkung auf die osmotische Resistenz zuzuschreiben.

Im übrigen war zu beobachten daß der Gesamtfettgehalt des Blutes die Wirksamkeit des Oleats beeinflußt. Es zeigte sich nämlich, daß bei lipämischem Blut höhere Oleat Konzentrationen erforderlich sind, um denselben stabilisierenden Effekt an den Erythrozyten wie im Normalblut zu erzielen.

Morphologisch ist an den Erythrozyten nach Zusatz von UFS oder deren Salzen eine Mikroplanie, später Stechapfelformen und schließlich kurz vor der Hämolyse eine Sphärozytose festzustellen. Der Hämatokritwert bleibt bei allen diesen morphologischen Veränderungen der Erythrozyten konstant es ändert sich also nur die Gestalt, nicht aber das Volumen der Erythrozyten. Die mechanische Resistenz der Erythrozyten bleibt bei Konzentrationen bis zu 60 mg Na Oleat innerhalb des Normbereichs (ca 6 %) (2)

Dagegen ändert sich die Inkubationshämolyse unter Zugabe von Na-Oleat gegenüber der Norm deutlich. Während sichere Effekte bis 48 Stunden nicht beobachtet werden konnten, kommt es nach 96 Stunden zu einer deutlichen Hämolysehemmung mit steigenden Oleat Konzentrationen bis 60 mg % (Abb. 2)

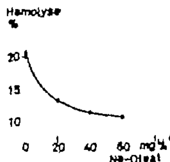


Abb. 2. Die Inkubationshämolyse von Erythrozyten bei 37 °C nach 96 Stunden in Abhängigkeit von der Konzentration an Natrium-Oleat.

b) Vergleichende Untersuchungen verschiedener Fettsäuren. Verschiedene UFS beeinflussen die osmotische Resistenz verschieden stark. Äquimolare Mengen Ölsäure, Linolsäure und Linolensäure zeigen eine im Prinzip gleiche, jedoch verschieden starke Wirkung auf die osmotische Resistenz. Bei vergleichbaren, niederen Konzentrationen nimmt das Ausmaß der Verbesserung der osmotischen Resistenz bei der Linolsäure und noch mehr bei der Linolensäure ab bei vergleichbaren, hohen Dosen ist die Hämolysewirkung in der Reihenfolge Ölsäure – Linolsäure – Linolensäure größer (Abb 3) Auch eine nur in 40 %iger Reinheit vorliegende Arachidonsäure zeigte eine positive Wirkung auf die osmotische Resistenz.

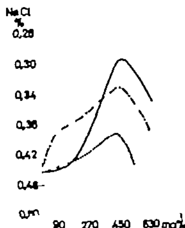


Abb. 3. Osmotische Resistenz von Erythrozyten. 50% Hämolysewerte in Abhängigkeit von der Konzentration von Ölsäure (——), Linolsäure (– – –) und Linolensäure ( )

Stearinsäure, Palmitinsäure und deren Natrium-Salze konnten nicht in vergleichbaren Konzentrationen im Serum gelöst werden qualitativ zeigten auch diese Substanzen ähnliche Effekte.

c) Die osmotische Resistenz nach Zusatz verschiedener Lipide. Entsprechend der Beobachtung, daß lipämische Seren keine erhöhte osmotische Resistenz zeigen, konnte auch bei Zusatz von Neutralfetten, wie Olivenol, und essentiellen Phospholipiden in vergleichbaren Konzentrationen keine osmotische Resistenzerhöhung der Erythrozyten beobachtet werden.

d) Der Einfluß körperfremder Substanzen auf die osmotische Resistenz von Erythrozyten Durch Vergleich der UFS mit verschiedenen Substanzen ähnlicher physikochemischer Eigenschaften wie UFS versuchten wir zu klären, ob irgendwelche unspezifische Effekte der Fettsäuren zur Erhöhung der osmotischen Resistenz beitragen.

Als ein möglicher, unspezifischer Effekt der Fettsäuren könnte die Mizellbildung, eventuell die positive oder negative Ladung dieser Mizellen, betrachtet werden. Wenn der Erhöhung der osmotischen Resistenz dieser Mechanismus zugrunde läge, dann müßten Substanzen wie Dodekansulfonat oder Cetyltrimethylammoniumbromid, von denen die eine negativ geladene, die andere positiv geladene Mizellen bildet, einen ähnlichen Effekt wie die UFS auf die Erythrozyten ausüben. Das aber war nicht der Fall. Beide Testsubstanzen zeigten bei niedrigen Konzentrationen keine sichere Verbesserung der osmotischen Resistenz bei hohen Dosen erfolgte Hämolyse. Oberflächenaktivität ohne Mizellbildung konnte bei Digitonin erwartet werden. Auch hier zeigt ein Digitoninzusatz in steigender Konzentration eine zunehmende Verschlechterung der osmotischen Resistenz bis zur Hämolyse.

Daß die drei besprochenen Substanzen keine osmotische Resistenzerhöhung bei Erythrozyten erzeugen können, obwohl ihr allgemeines, physikochemisches Verhalten den UFS sehr nahe kommt, deutet darauf hin, daß die UFS durch einen substanzspezifischen Effekt die Resistenzerhöhung der Erythrozyten auslösen.

Diskussion

Die unveresterten Fettsäuren des Blutes sind vorwiegend an Albumin und a_1 Globulin gebunden (1) und sind die metabolisch aktivste Lipidfraktion. Die Mittelwerte der UFS werden von verschiedenen Autoren (4 5 6) mit 8,8 bis 17 mg% angegeben. Während die Bedeutung der UFS im Lipidstoffwechsel in den letzten Jahren immer mehr hervortrat, ist über ihre Wirkungen auf den Erythrozyten bisher wenig bekannt geworden.

Die bisherigen Untersuchungen ergaben, daß beim Zusatz von UFS zum Blut die Form (7) und die osmotische Resistenz der Erythrozyten (1 7) sowie die Blutsenkungsgeschwindigkeit (BSG) verändert werden (8, 9)

Der Effekt der Verbesserung der osmotischen Resistenz UFS-inkubierter Erythrozyten ist konzentrationsabhängig, wobei als wirksames Agens nicht die freie Fettsäure, sondern deren Salz verantwortlich sein dürfte. Beachtenswert ist die Tatsache daß ein und die gleiche Substanz bei niederer Dosierung eine Hemmung der Hämolyse bewirkt, jedoch bei höherer Dosierung die Erythrozyten lysiert. Auffällig ist auch das Verhalten der ungesättigten

C_{18}-Fettsäuren (Ölsäure, Linolsäure, Linolensäure). In vergleichbaren Mengen nimmt mit zunehmender Anzahl an Doppelbindungen die resistenzerhöhende Eigenschaft ab und die Hämolysewirkung bei höheren Dosen zu.

Die UFS beeinflussen auch die mechanische Resistenz und die Inkubationshämolyse im Sinn einer Herabsetzung der Hämolyserate.

Andere oberflächenaktive, fettsäureähnliche und Mizellen bildende Stoffe, so das Dodekan-Sulfonat und das Cetyltrimethylammoniumbromid ergeben bei äquimolaren Mengen keine signifikante Erhöhung der osmotischen Resistenz.

Die Frage nach dem Wirkungsmechanismus der UFS auf die anfängliche Erhöhung der osmotischen Resistenz und die nachfolgende Hämolyse bei steigenden Konzentrationen muß offen bleiben. Es ist zu vermuten, daß es sich um einen substanzspezifischen Effekt der UFS handelt, der nicht mit der Eigenschaft der Oberflächenaktivität der UFS in Zusammenhang steht.

Die gleichzeitig auftretenden morphologischen Änderungen der Erythrozytenform sind unspezifisch. Sie treten bei Schädigung der Erythrozyten durch oberflächenaktive Stoffe ebenso auf wie etwa auch bei immunopathologischen Alterationen (10). Offenbar handelt es sich dabei um ein allgemeines Epiphänomen.

Überraschenderweise ergab sich auch bei der Untersuchung einer Ovalozytose, daß Oleat in der Lage ist, die pathologische Erythrozytenform zu normalisieren und gleichzeitig die osmotische Resistenz zu erhöhen. Da schon kleine Mengen zugesetzten Natrium-Oleats zum Blut in Konzentrationen, wie sie physiologischerweise vorkommen können, eine Verminderung der Hämolyse hervorrufen, ist zu diskutieren, ob nicht auch die im Blut in vivo befindlichen UFS im Sinn eines Hämolyseschutzes wirksam werden. Darüber hinaus ergeben diese Versuche, daß auch bei höheren Fettsäurekonzentrationen, wie sie pathologischerweise auftreten können – bis etwa 100 mg/ (4) – die Schutzfunktion erhalten bleibt.

Zusammenfassung

Unveresterte, freie Fettsäuren (UFS) beeinflussen die Hämolyse menschlicher Erythrozyten. Neben einer Erhöhung der osmotischen und mechanischen Resistenz wird auch die Hämolyserate bei der Inkubationshämolyse herabgesetzt. Die physiologische und pathologische Bedeutung dieses Effektes wird diskutiert.

Summary

Unesterified free fatty acids influence the human red cells. The osmotic and mechanic resistance increases, the haemolysis during incubation is reduced. Some physiologic and pathologic aspects are discussed.

Résumé

Les acides gras libres non estérifiés ont une influence sur les erythrocytes humains. La résistance osmotique et méchanique augmente, l'hémolyse pendant incubation est diminuée. Quelques aspects physiologiques et pathologiques de cette influence sont discutés.

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Acta haemat. 32. 335–337 (1964).

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A Modification of the Hicks and Pitney Test for the Distinction between Haemophilia A and Christmas Disease

By C. Gardikas, G. Arapakis, D. Thomopoulos and T. Kanaghinis

The thromboplastin generation test devised by Biggs and Douglas (1) is the most reliable test for the differentiation of haemophilia A from Christmas disease. On the other hand, the screening test described by Hicks and Pitney (3) provides a rapid and sensitive means of detecting the abnormalities of thromboplastin generation, but being non-specific it cannot distinguish between haemophilia A and Christmas disease. Hence, when the screening test gives abnormal results, one has to proceed further to the performance of the orthodox thromboplastin generation test.

In the present paper a modification of the Hicks and Pitney test is described which enables the distinction to be made between haemophilia A and Christmas disease.

Material and Methods

Principle: When with the usual Hicks and Pitney incubation mixture the substrate clotting time is prolonged, the patient has either haemophilia A or Christmas Disease. If second incubation mixture containing in addition diluted normal serum gives normal clotting time, the plasma in the incubation mixture is deficient in Christmas factor; if not the plasma is deficient in factor VIII.

Reagents:) Platelet suspension prepared as described by Biggs and Macfarlane (2) b) citrated plasma prepared as described by Hicks and Pitney diluted 1 10 with imidazole buffer pH 7.3, c) imidazole buffer prepared as described by Biggs and Macfarlane (2), d) normal serum prepared as described by Biggs and Macfarlane and diluted 1 10 with imidazole buffer) substrate plasma prepared as described by Hicks and Pitney and f) 0.025 M-$CaCl_2$.

Procedure. Two incubation mixtures are prepared. The first consists of equal volumes of platelet suspension, plasma to be tested, diluted 1 10 imidazole buffer and solution of $CaCl_2$, in the second imidazole buffer is substituted by equal volume of normal serum, diluted 1 10 The clotting times of the substrate at the 4th and 5th minute are recorded. In details the technique of Hicks and Pitney is followed. A parallel incubation mixture containing normal plasma, diluted 1 10, is used as control.

Acta haemat. 32: 348–367 (1964)

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Studies of Basophils

Basophilic Leukocytes and Gonadal Activity in the Rabbit*

By Ernst Thonnard-Neumann

Clinical and experimental evidence indicates that the economy of basophilic leukocytes is subject to control by ovarian hormones. Their number in the circulating blood increases under the influence of estrogens and decreases under the action of progesterone (1 2). I have found (3) that young women have higher basophil numbers than elderly women or young or old men. Among rabbits, mature females have higher basophil means than immature females or male animals. Furthermore women and female rabbits have a greater proportion of young basophils than their male counterparts.

The present study deals with observations on basophils during various states of gonadal function in the rabbit.

Materials and Methods

Fourty-four rabbits of mixed breeds were used. They were fed standard diet and kept in individual cages. During the greater part of the study the cages were in air conditioned quarters, the remaining time they were in the open. Almost daily white cell and basophil counts were made over periods lasting from several weeks to 4 years. Blood was taken from marginal ear veins. Total leukocyte counts were made in conventional chambers or with the Coulter Counter. Basophils were counted in Speirs-Levy chambers after the blood had been diluted with solution of the following composition 0.05% aqueous solution of toluidine blue in 1.5% acetic acid. The average of two chamber counts was used. Blood smears were stained with Wright-Giemsa, May-Gruenwald-Giemsa or Undritz toluidine blue. In the smears the basophils were differentiated according to number size and position of the granules. Each of these characteristics was classified into three groups numbers as numerous, medium and few sizes as coarse, medium and small positions as peripheral ("P") intermediate ("I") and uniform ("U"). In the "P" form the granules were in peripheral position at one side of the cytoplasm and the round nucleus at the other side. In the "I" form some granules traversed the cytoplasm between the lobes of the usually two lobed nucleus and in the "U" type

* This investigation was supported by USPHS Research Grant HE 07362 from the National Heart Institute, Public Health Service, Bethesda, Maryland, USA.

basophil the granules covered the cell surface in more or less uniform manner thereby frequently obscuring the multilobed nucleus. The P form was considered as the youngest and the U form the most mature basophil normally found in the peripheral blood (fig. 1)

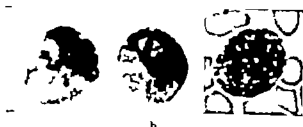


Fig 1 Rabbit basophils) peripheral P form, b) intermediate T form,) uniform U form.

Basophils were studied in isolated rabbits and during ovulation, pregnancy and pseudopregnancy after castration and estrogen treatment. In view of the considerable differences in numbers of white cells and basophils between individual animals each rabbit served as its own control. Overall means were calculated directly from the means of the individual animals. Data analysis during ovulation, pregnancy and pseudopregnancy was made by analysis of co-variance, after castration and estrogen therapy by Wilcoxon Rank-Sum-Test.

If not stated otherwise the term statistical significance refers to the ninety-five percent level of confidence.

Results

Ovulation

In the rabbit ovulation occurs about 10—12 hours after copulation. Twenty previously isolated estrous does were mated with either intact or vasectomized bucks. One doe received an intravenous injection of 500 units of chorionic gonadotropic hormone.

Ovulation in 19 animals was verified either by the resulting pregnancy or pseudopregnancy or by inspection of the ovaries. Precopulation basophil levels were established from numerous counts made in the weeks or months preceding mating On the day of copulation counts were made immediately before and 10—12 hours afterwards. In 15 does an a erage drop of 41 / (range 6—94) below the precopulation means was seen In 3 animals a rise and in one no change occurred (fig 2 and 3)

Pregnancy

In 7 pregnant rabbits almost daily counts were made No significant change in mean basophil numbers from those found in

the same animals before pregnancy were observed. However on two separate days basophil numbers rose in all animals suddenly and significantly above the mean level In the majority of the observations the rises lasted only one day The first elevation occurred between the 12th and 18th day of pregnancy the second between days 27 and 29 On the day of parturition the numbers dropped and remained low for 1—3 days post partum. Changes in basophil numbers occurred independently of variations in the total white cell count (fig 2)

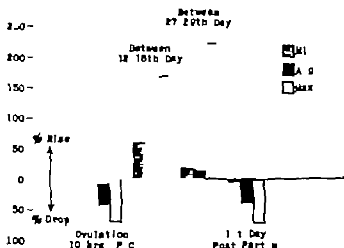


Fig 2 Changes of basophil numbers during pregnancy expressed in abov and below the mean (7 rabbits)

The morphology of basophils differed in the pregnant animals from that seen in the isolated estrous does. The changes involved position and size of the granules and to a lesser extent their numbers. Pregnant rabbits had a higher proportion of mature basophils

Table I
Effect of pregnancy on position, number and size of granules of basophilic leukocytes (expressed in % of 2450 cells)

	Position			Number			Size		
	P	I	U	num.	med.	few	coarse	med.	small
5 estrous rabbits	8.5	25.4	66.1	18.5	73.0	8.	16.7	66.1	17.2
5 pregnant rabbits	6 0	17 7	76.3	19 6	77 4	3.0	13.3	73.1	13 6
5 male rabbits	6.6	18.1	75.3	16 6	77.6	5 8	11 7	75.3	13 0

The differences between estrous and pregnant rabbits are significant for each group ($P < 0.001$)

(U' type) a greater number of cells with numerous granules and less variation in the size of granules (table I)

Pseudopregnancy

Sterile coitus in the rabbit induces ovulation followed by progestational changes which last about sixteen days. Almost daily counts were made during such a state of pseudopregnancy in 8 rabbits who had been mated with sterile bucks. In two rabbits the mean absolute numbers of basophils were significantly lower than before pseudopregnancy In the other 6 animals insufficient counts had been made before mating took place. Between days 12 and 15 the number of basophils rose in all animals for one day The average increase was 75 / with a range from 16—146 / (fig 3)

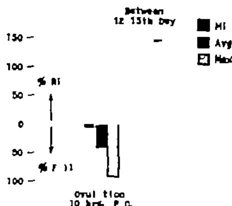


Fig 3. Changes of basophil numbers during pseudopregnancy (8 rabbits)

Oophorectomy

The ovaries of 6 mature rabbits were removed under general anesthesia For 3 to 4 days after the operation absolute and relative basophil numbers were low They rose again after this time to approximately preoperative levels and remained so for about 2 weeks. Basophil percentages dropped in all animals while absolute numbers dropped in 3 and rose in the other 3 during the following 8 months. From the 9th month absolute and relative basophil numbers became low in all animals and remained below 50 / of the preoophorectomy mean. Longest period of observation was 25 months.

Estrogen Therapy

18 mature rabbits, 10 of them intact and 8 oophorectomized, were treated with various estrogen preparations. Estradiol benzoate in oil was given intramuscularly, estrone and conjugated estrogens in aqueous solution intravenously. Smallest individual dose was 0.2 mg, largest 2.0 mg. Smallest amount given in one series was 1.5 mg and largest total dose 30 mg. These were given in several series of maximum 10 mg each which were repeated every 4 to 5 months.

Immediate effects. Beginning 15 minutes after the first injection several counts were made during the first 24 hours. In 113 counts considerable fluctuations of the total white cell and basophil count were seen. The changes were inconsistent but a tendency for increased basophil numbers was observed after higher doses (2.0 to 2.5 mg estrogens) had been given.

Intermediate effects. In 235 counts made during the next 2 weeks, no consistent changes of the total white cell numbers were seen. The absolute and relative basophil counts, on the other hand, rose significantly in all animals after an average of 9 days.

Long range effects. In 12 rabbits, 6 intact and 6 oophorectomized, treatments and blood examinations were continued until the total dose of 30 mg estrogens had been reached. Frequent counts were made for another 16 months after the end of treatment. A total of 712 blood samples were examined. An increase of the white cell numbers above 18 000 per mm³ was repeatedly observed in 7 rabbits. The absolute and relative basophil counts rose in all 12 animals. Higher basophil numbers were seen in about 50 % of the leukocytotic episodes. The increase in basophil numbers were proportionally higher than those of the total white cell numbers. However, in the great majority of the observations no correlation between changes in basophil and total leukocyte numbers was seen. In oophorectomized rabbits the mean rise of absolute basophil numbers was 146 % and of relative numbers 220 % above pretreatment postoophorectomy means. The corresponding increases for intact animals were 91 and 86 %. In 10 of the 12 rabbits these differences were significant ($P < 0.02$). In some oophorectomized rabbits the elevation persisted 8 months after the end of therapy.

In an intact but estrogen deficient rabbit changes in basophil maturity were observed while the animal received a total of 2.7 mg of estradiol. The absolute number of basophils rose from a pretreatment mean of 265 basophils per mm³ to one of 377; at the same time the proportion of young P forms increased by 100 %. 33 days

later the absolute number of basophils and the proportion of P' forms had returned to pretreatment levels.

Cyclic Fluctuations

Basophil numbers. Sudden spontaneous rises of basophil numbers occurred in all rabbits. They were independent of the fluctuations of the white cell count. The peaks lasted usually only one day. Among a total of 178 significant elevations above the mean number of basophils a cyclic regularity was observed in 142 instances. 86 times the intervals between two peaks were 7 days or multiples thereof. 56 times they were 7±1 or multiples of 7±1 days apart. 36 times there was no regularity. The findings are summarized in table II.

Table II

Intervals between peaks of rabbit basophil numbers during various states of gonadal function.

	No. of rabbits	Intervals 1	2	3–4	Irregular	Total
		weeks				
Isolated adult males	5	10	4	3	7	24
Isolated estrous females	15	25	5	7	5	42
Immature males	3	2	3	–	2	7
Immature females	8	2	7	3	4	16
Pregnant females	6	1	5	1	4	11
Pseudopregnant females	8	3	5	–	4	12
Oophorectomized females	7	—	4	8	7	19
Estrogen treated intact females	6	13	7	2	2	24
Estrogen treated oophorectomized females	7	12	8	2	1	23
	Total	regular 142		Irregular 36		178

Vaginal cytology. When vaginal smears were obtained from the upper part of the vagina with a method described by HAMILTON (4) cyclic changes in the maturity of the epithelium could be observed. In 6 estrous rabbits a higher percentage of superficial vaginal cells with pycnotic nuclei was found every 5—8 days with an average of 7 days. On 14 occasions high basophil numbers and a high percentage of superficial vaginal cells were found on the same day. 11 times they occurred within one day from each other and 10 times there was no coincidence.

Seasonal Changes

The basophil numbers of rabbits kept in cages in the open dropped significantly during the hot weather season. The counts

rose again when the animals were transferred to air conditioned quarters. No significant variations of the total leukocyte counts were seen during these changes.

Discussion

Ovulation. The reduction in basophil numbers subsequent to ovulation confirmed previous reports by BOSEILA on the rabbit (5) and by me on humans (6) In normal women the middle of the menstrual cycle frequently is marked by a sudden drop of basophil numbers. The reduction coincides with the characteristic lowering of body temperature which precedes ovulation. Since this is the time of maximum secretion of luteinizing hormone by the anterior pituitary it would seem possible that the drop in basophil numbers took place either under the direct influence of LH or through the action of newly formed progesterone. A third explanation would be that the stress of ovulation caused an increase in adreno-cortical activity and thereby basopenia as corticotropin and cortisone are known to lower the number of circulating basophils (1)

Pregnancy In the rabbit progesterone is indispensable for the maintenance of pregnancy by preventing premature myometrial contractions (7) By day 29 that is 2—3 days before parturition, there is a sharp increase in estriol excretion and the progesterone block against myometrial contractions is withdrawn (8) This is also the time of the second peak in basophil numbers. Not much information is available on the state of hormonal balance at mid-pregnancy the time when the first rise in basophil numbers occurs. There exists, however experimental evidence that a progestational state induced by progesterone or chorionic gonadotropin cannot be extended beyond 17 ± 2 days without progestational activity breaking down for 48 hours. With continued treatment the progestational state can again be induced for one or more phases of similar length (9 10) It would therefore, seem possible that the increase in basophil numbers during midpregnancy was brought about by a similar shift away from progesterone dominance as the rise which preceded parturition

The drop in basophil numbers which followed upon delivery may be explained by the loss of estrogens which were expelled with the placentas and the increase in some corticosteroids which accompanies early puerperium (11)

Pseudopregnancy The changes which characterize the uterus of the pseudopregnant rabbit closely resemble those of the first half of pregnancy (7) Pseudopregnancy is maintained by the secretory activity of the corpus luteum. This comes to an end by day 14. Meanwhile excretion of estrogen has risen to a maximum level, the endometrium becomes congested and there may be bleeding into the stroma. The rise in basophil numbers preceding the end of pseudopregnancy coincided with the shift in hormonal balance from progesterone to estrogen.

Oophorectomy and therapy The basophilic leukocytes were the only white blood cells which showed a significant numerical reduction after oophorectomy This would seem to emphasize their close relationship with ovarian function. The period of delay before a lasting drop was observed may be explained by the slowly decreasing levels of ovarian hormones after oophorectomy This view is further supported by the persistence of higher basophil numbers for several months after estrogen therapy was finished. It seems also noteworthy that the rises were proportionally higher in the operated than in the intact rabbits.

Cyclic variations. Several manifestations of gonadal activity in the male and female rabbit change in a cyclic manner Alterations of the vaginal epithelium and the ejaculate appear to have cycles varying between 3 and 8 days (4 12 13) In the present studies the prevailing interval between spontaneous rises of basophil numbers was seven days. During pregnancy and pseudopregnancy frequently a two week rythm was seen. Intervals were longest in the immature and the oophorectomized animals. Estrogens made the cycles short and more regular Maturation cycles of the vaginal epithelium were about one week long. There was a fairly high degree of positive correlation between a mature vaginal cell picture and high basophil numbers.

Seasonal changes The observations when no peak in basophil numbers was seen while an estrogenic type of vaginal epithelium existed were made during the hot weather season while the rabbits were in cages in the open and the general level of basophil numbers among them was low The decrease in the basophil count during the summer months could be a manifestation of heat adaptation which produced an increased release of adreno-cortical steroids and thereby a basopenia. On the other hand the low level of basophil numbers might have been a sign of reduced gonadal activity since,

according to ASDELL (14) summer is the worst time for breeding rabbits in the United States.

Conclusion

The results of the present studies confirm and supplement previous findings on the influence of gonadal hormones on the number of circulating basophils. The observation of morphological alterations in the basophils provide an additional insight into their maturation and their release from the bone marrow under the control of these hormones. Little is known about the fate of basophils which disappear from the blood stream under physiologic conditions. Migration of basophils from the peripheral blood to the genital organs of the ovulating rabbit is suggested by observations of ZACHARIAE et al. (15) and the writer (unpublished data) They found in the ovaries around the mature follicles an accumulation of basophils while their number in the circulation had dropped. A positive correlation between estrogen activity and uterine mast cell contents has been observed in humans, guinea pigs and mice (16, 17 18) These species have numerous tissue mast cells while the rabbit has few but an abundance of blood basophils. It would seem a reasonable contention that under similar stimuli blood basophils are capable of taking over the same functions in tissue as mast cells.

Summary

In the rabbit the number of basophilic leukocytes varies with the state of gonadal function. Changes observed during ovulation, pregnancy and pseudopregnancy indicate that basophil numbers increase under estrogen and decrease under progesterone domination. Oophorectomy produces lasting drop in basophil numbers; they rise after estrogen therapy Spontaneous rises in basophil numbers occur in cycles of about 7 days or multiples thereof. Short cycles predominate in the estrous, long cycles in the oophorectomized female. Determined by the proportion of young cells, estrous females has more rapid turnover of basophils than either pregnant does or mature male rabbits.

Résumé

Chez le lapin le nombre des leucocytes basophiles arie selon la phase fonctionnelle des gonades. Les changements pendant l'ovulation, la gravidité et la pseudogravidité montrent que le nombre des basophiles augmente sous l effet des oestrogènes et diminue sous celui de la progestérone. L oophorectomie produit une diminution durable du nombre des basophiles il remonte après un traitement à l'oestrogène. Des augmentations spontanées du nombre des basophiles apparaissent par périodes de 7 jours ou de plusieurs fois cette durée. Les cycles courts prédominent durant les périodes de forte production d'oestrogène, les cycles longs chez les femelles dont les ovaires ont été extirpés. D après la proportion de cellules jeunes les femelles ont durant les phases oestrogènes un turnover des basophiles plus rapide que les femelles gravides ou que les lapins mâles adultes.

Zusammenfassung

Beim Kaninchen variiert die Zahl basophiler Leukozyten mit der funktionellen Phase der Gonaden. Die Veränderungen bei Ovulation, Gravidität und Pseudogravidität zeigen, daß die Basophilenzahl bei Oestrogenwirkung ansteigt und bei Progesteronwirkung abnimmt. Oophorektomie führt zu einem dauernden Abfall, während nach Oestrogentherapie ein Anstieg erfolgt. Spontane Steigerungen der Basophilenzahl treten in Perioden von 7 Tagen oder Mehrfachen davon auf. Kurze Perioden überwiegen bei Weibchen im Oestrus, lange Perioden bei oophorektomierten Weibchen. Beurteilt nach dem Anteil jugendlicher Zellen haben Weibchen im Oestrus einen rascheren Basophilenumsatz als gravide Weibchen oder erwachsene Männchen.

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